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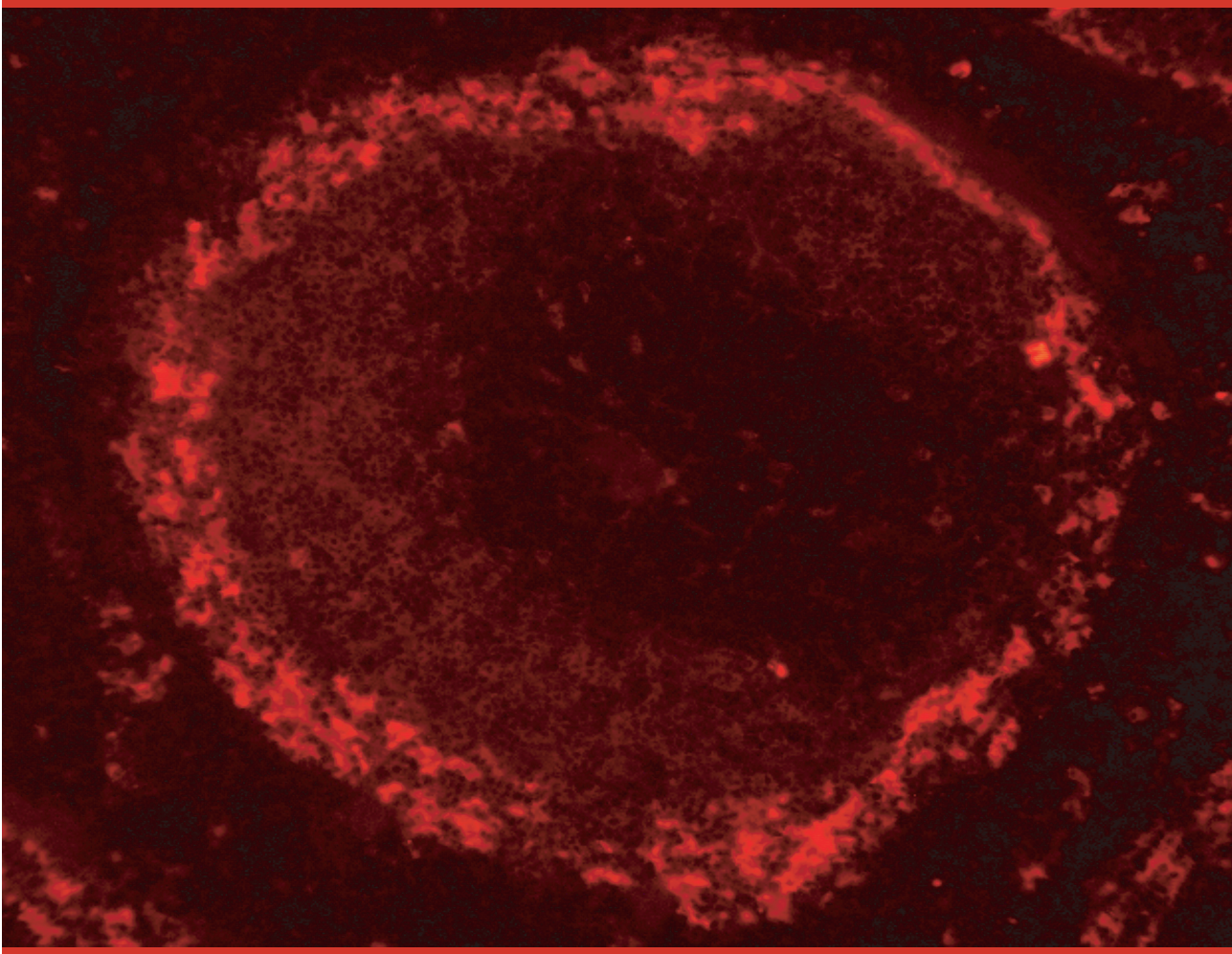

E.A. Koppel

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(E.A. Koppel)

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The in vivo function of mSIGNR1, a DC-SIGN homologue

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door

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geboren te Rotterdam

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copromotor:	dr. T.B.H. Geijtenbeek

"It was the best of times,
it was the worst of times…"

(Charles Dickens,
A Tale of Two Cities)

Contents

List of abbreviations

AraLAM	LAM lacking a mannose cap
CFU	Colony-forming units
CLR	C-type lectin receptor
CRD	Carbohydrate recognition domain
CWPS	Cell wall polysaccharide
DC	Dendritic cell
DC-SIGN	Dendritic cell-SIGN
GPI	Glycosylphosphatidylinocitol
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
ICAM	Intercellular adhesion molecule
IFN	Interferon
IL	Interleukin
KO	Knock-out; deficient for
LAM	Lipoarabinomannan
Le	Lewis
LPS	Lypopolysaccharide
LSEC	Liver-/lymph node sinusoidal endothelial cell
LSECtin	LSEC C-type lectin
L-SIGN	Liver-/lymph node-SIGN
ManLAM	Mannose-capped LAM
MARCO	Macrophage receptor with a collagenous structure
MMM	Marginal metallophilic macrophage
MR	Mannose receptor
mSIGNR1	Murine SIGN related 1
MZB	Marginal zone B cell
MZM	Marginal zone macrophage
PAA	Polyacrylamide

PC	Phosphorylcholine
PS	Polysaccharide
Siglec-1	Sialic acid-binding immunoglobulin-like lectin-1
SIGN	Specific ICAM-3-grabbing nonintegrin
Th	Helper T cell
TI	T-independent
TLR	Toll-like receptor
TNF	Tumor necrosis factor
WT	Wild-type

Chapter 1

The in vivo function of mSIGNR1, a DC-SIGN homologue
General introduction

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1.1 The spleen

The spleen is the largest secondary lymphoid organ in the mammalian body and exerts a large array of functions. The spleen maintains the erythrocyte population, captures blood-borne pathogens and facilitates the induction of both innate and adaptive immune responses[1]. The unique structure of the spleen facilitates the plethora of functions the spleen exerts. In Box 1 the anatomy of the spleen is discussed. The immune system has developed several mechanisms to combat pathogens. The T cell dependent humoral immune response, which involves the activation of B cells by T cells and induces high affinity antibody production and life-long memory, takes place in lymph nodes and the white pulp of the spleen, and plays a key role in the destruction of extracellular pathogens. However, this mechanism needs more than one week to become fully effective. There are numerous pathogens that have the capacity to cause severe pathology and even death within this time. Therefore there are other mechanisms within the immune system that are active early after pathogen encounter. One of these mechanisms is independent of T cells and is called the T-independent (TI) immune response. TI antigens can be divided into 2 groups. TI-I antigens, such as lipopolysaccharide (LPS) , are polyclonal stimulators that are totally independent of T cells in evoking a response, whereas TI-II antigens, such as Ficoll, the *Streptococcus pneumoniae*-epitope phosphorylcholine (PC) and dextran, are independent of T cell help but still need T cell derived factors[2–5]. TI-II antigens are molecules with multiple, repeating subunits and upon intravenous administration target the marginal zone of the spleen[6,7]. Several studies have been performed to investigate the role of cells in the marginal zone of the spleen in TI immune responses. It has been described that marginal zone B cells (MZB) are pivotal to the TI immune response against the model TI antigen Ficoll[8]. In addition, marginal zone macrophages (MZM) are also involved in the TI immune response as they capture TI-II antigens and the anti-Ficoll response is diminished upon elimination of MZM by liposomes[9]. Hence both MZM and MZB are crucial to the TI-II immune response. However, depletion of MZM using ERTR-9 for targeting, did not result in a decrease in the TI immune response, suggesting that the function of the MZM in the marginal zone is less straightforward[10,11]. Still, the unique situation of this cell population in the marginal zone that enables the capture of blood-borne pathogen and the close proximity to MZB cells point towards the involvement of MZM in the TI immune response[12].

Recently it was discovered that the MZM-specific monoclonal antibody ERTR-9 recognizes a murine specific intercellular adhesion molecule- (ICAM-)grabbing nonintegrin related 1 (mSIGNR1)[13,14] . mSIGNR1 is a C-type lectin that is exclusively expressed by MZM and is neither expressed by marginal metallophilic macrophages (MMM) nor the splenic red pulp macrophages. It is one of five murine homologues of the human C-type lectins dendritic cell-specific intercellular adhesion molecule 3 (ICAM 3)-grabbing nonintegrin (DC-SIGN) and liver/lymph node-SIGN (L-SIGN) that play important roles during homeostasis and pathogen recognition.

Box 1: The spleen

The anatomy and vascularization of the spleen

The spleen has a central position in the blood circulation and has an ingenious vascularization. The afferent splenic artery enters the spleen in the region of the hilus and branches into a number of central or penicillar arterioles that terminate as end arterioles in the red pulp (Fig. 1.1). Central arterioles branch into numerous small follicular arterioles in the white pulp [15–17]. These lymphoid areas have a white appearance at the macroscopic level and are therefore referred to as the white pulp. The white pulp follicles display a high resemblance to lymph nodes, since in both lymphoid organs B cells are located in follicles surrounded by a T cell area where the central arteriole is located. This anatomical organization facilitates an optimal interaction between T and B cells, which is pivotal for the induction of a potent immune response.

The red pulp surrounds the white pulp areas and consists of venous sinuses and pulp cords (Fig. 1.1). The red pulp mediates the filtering of the blood by the spleen. The cords contain fibroblasts that maintain a network of reticular fibers. In addition, numerous macrophages are found within this area of the spleen. In contrast to the cords, which form an open blood system and thus are devoid of any endothelial lining [18], the venous sinuses are lined by a discontinuous layer of endothelial cells oriented along the longitudinal axis of the sinus. These endothelial cells contain stress fibers and are kept together by annular fibers, which are part of the extracellular matrix. Upon contraction of the stress fibers, an intercellular space appears. Blood enters the pulp cord by terminal arterioles and is filtered by the passage through the network of reticular fibers in the cord and the passage through the endothelial slits surrounding the venous sinuses (Fig. 1.1).

Moreover, the quality of the erythrocyte population is monitored; “old” erythrocytes, which have a rigid membrane, are not able to deform and penetrate the slits between the endothelial cells and are phagocytosed by the numerous macrophages residing in the cords. In addition, erythrocytes bearing intracellular inclusions or intracellular organisms such as malaria can be cleared from these components during the passage without destruction of the erythrocyte. The membranes of the cells close and the cells pass into the sinuses while the phagocytic cells will clear the inclusions and the aged erythrocytes [19,20]. Hence, the red pulp of the spleen functions as an ingenious filter system pivotal for the quality of the erythrocyte population and the clearance of intracellular inclusions. As described, a large part of the arterial blood traverses the white pulp, reaches the venous sinuses and leaves the spleen. However, a proportion of the terminal arterioles ends in a sinus in a distinguishable concentric area that separates the white and red pulp; the marginal zone [21,22]. In contrast to mice, which have one marginal zone, humans have an inner and an outer marginal zone, which is surrounded by a large perifollicular zone. The blood flow in the marginal sinus is low and the marginal zone is an important transit area for recirculating B and T cells that are leaving the bloodstream and entering the white pulp [23] (Fig. 1.1). In addition to being a transit area, the marginal zone contains a large number of resident cells. These cell populations are marginal metallophilic macrophages (MMM), marginal zone B cells (MZB) and marginal zone macrophages (MZM) (Fig 1.1). Together these cells are involved in the screening of the blood for pathogens, debris and foreign particles.

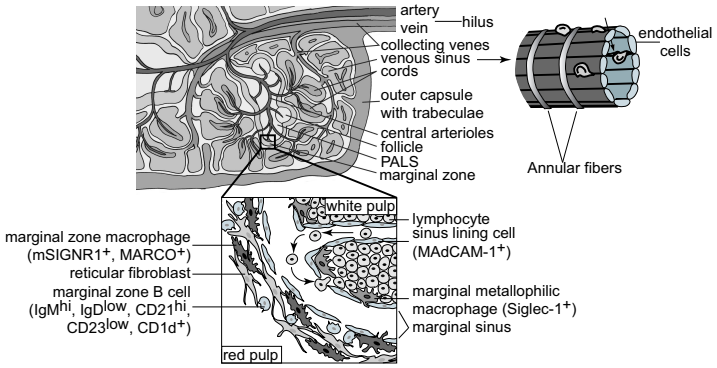

Figure 1.1: The anatomy and vascularization of the spleen
Schematic overview of the structure and vascularization of the spleen based on the figures drawn by G. Kraal [1].

Cells in the marginal zone of the spleen

Between the white pulp and the sinus, sialic acid-binding immunoglobulin-like lectin-1 (Siglec-1) expressing MMM are situated [24] (Fig. 1.1). MMM display a high affinity for silver and hence are called metallophilic cells, and are strategically positioned along the borders of the marginal sinus, ideal for the uptake of blood-borne antigens [16]. However, in spite of their acid phosphatase activity and ability to take up iron compounds, they do not phagocytose well [16].

At the outer face of the marginal sinus, bordering the red pulp, the MZM reside (Fig. 1.1). MZM are big cells with long cell processes. These cells form a ring of two or three layers dispersed throughout the marginal zone. They are highly phagocytic, and often contain, when examined at the ultrastructural level, phagolysosomes with necrotic erythrocytes and cellular debris [25]. This unique macrophage subpopulation is characterized by the expression of the macrophage receptor with a collagenous structure (MARCO) and ERTR-9 antigen [25,26]. Upon systemic administration of bacteria or LPS, MARCO expression is induced within the red pulp macrophage population whereas ERTR-9 staining remains a specific marker for the MZM [27]. In contrast to other macrophages, MZM do not express MHC class II [7]. Consequently, MZM are not involved in the priming of T cells but may be involved in the activation of B cells. Indeed, MZM appear to have close contacts with a specific B cell population.

There are two splenic B cell populations; the mature recirculating follicular B cells are located in the B-lymphoid follicles in spleen and lymph nodes, and MZB, which can be found in the marginal zone. The MZB population comprises about 5% of all peripheral B cells. These cells are long-lived, apparently naive B cells with a partially activated phenotype [28–30]. MZB localize in the spleen to the marginal zone in the vicinity of the marginal sinus and in close proximity to the MZM, and do not readily recirculate [31]. MZB are in close approximation to the marginal sinus to facilitate interaction with blood-borne antigens (Fig. 1.1). MZB can be activated to become IgM-secreting plasmablasts within hours, which is vital to their function in the innate immune response [32]. Hence, MZB appear to constitute a first line of defense against blood-borne pathogens.

1.2 C-type lectins

C-type lectins are pathogen receptors expressed by antigen presenting cells, which are the sentinels of the immune system that sense danger signals such as infected or transformed cells and subsequently prime naive T cells to induce an adaptive immune response. Antigen presenting cells acquire antigen, and process this for presentation on MHC molecules to T cells and are hereby instrumental in the induction of T cell responses [33]. An important route of antigen uptake occurs through C-type lectin receptors that internalize antigens for processing and antigen presentation on MHC molecules by targeting the antigen to MHC II-positive late endosomes for degradation and subsequent antigen presentation [34,35]. C-type lectins contain one or multiple carbohydrate recognition domains (CRD) that enable them to bind to carbohydrate structures, which are present on the surface of many pathogens but are also expressed by self-antigens (Fig. 1.2). Thus, C-type lectins are not only involved in pathogen recognition but can also function in homeostasis [36,37]. Antigen binding to C-type lectins does not lead to immune activation, rather these receptors induce immune tolerance by default, and require simultaneous signaling through Toll-like receptors (TLR) that specifically recognize signature structures of pathogens to trigger pathogen-specific immune responses during infection [38,39].

The "SIGN" family of C-type lectins consists of DC-SIGN, L-SIGN and LSECtin (liver and lymph node sinusoidal endothelial cell C-type lectin) in human and the five homologues mDC-SIGN and mSIGNR1 to mSIGNR4 in mice. All SIGN members are type II C-type lectins that contain one CRD region that defines the ligand specificity of the receptor (Fig. 1.2).

1.2.1 The human SIGN molecules

Human DC-SIGN is located on chromosome 19p13 in close proximity to the CD23 gene. Two homologues L-SIGN (DC-SIGNR) and LSECtin are located adjacent to the DC-SIGN gene [40–42]. L-SIGN shares 77% amino acid sequence identity with DC-SIGN [40,42], whereas LSECtin has only 31% identity to DC-SIGN.

DC-SIGN (CD209) was the first SIGN molecule identified and found to be expressed at high levels on monocyte-derived dendritic cells (DC) [43] (Fig. 1.2). In situ, DC-SIGN is present on subsets of immature DC at dermal and mucosal sites in the periphery, and on both immature and mature DC in lymphoid tissue such as tonsils, lymph nodes, and spleen [43–45]. DC-SIGN is not expressed on DC subsets of blood, except for a subpopulation of $CD14^+$ cells with DC-like phenotype [35]. Other DC subsets such as plasmacytoid DC, and Langerhans cells do not express DC-SIGN.

Recently, it was shown that upon infection with *Mycobacterium tuberculosis* and *Mycobacterium leprae*, DC-SIGN expression is induced on alveolar and leprosy skin lesion macrophages with a tolerant phenotype respectively, suggesting expression of DC-SIGN on macrophage subsets [46,47].

The expression pattern of L-SIGN and LSECtin is strikingly different from that of

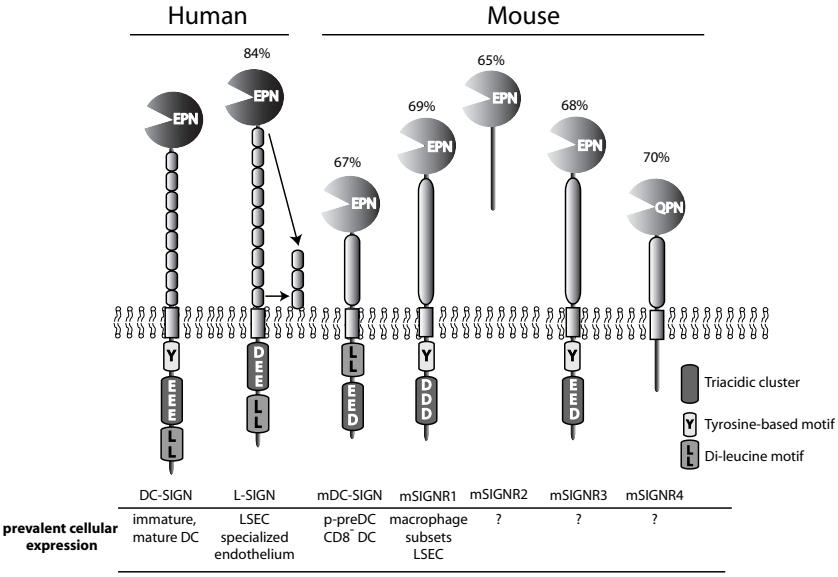

Figure 1.2: Schematic overview of the structure and expression of DC-SIGN and its human and murine homologues

All SIGN homologues are transmembrane receptors, except for mSIGNR2, which is a soluble receptor. The percentage amino-acid-homology of the CRD of the different homologues compared to DC-SIGN are depicted above the CRD. Within the CRD the highly conserved EPN sequence is essential for recognizing mannose-containing structures. All SIGN homologues contain the EPN motif, except for mSIGNR4, which has a QPN motif, indicating that mSIGNR4 may have another ligand specificity compared to the other SIGN molecules. In contrast to DC-SIGN that contains 7 complete and one incomplete repeat, the number of repeats of L-SIGN is variable and varies between 3 and 9 which is indicated with arrows. Within the cytoplasmic tail several internalization motifs are found. The di-leucin (LL) motif is thought to be important for internalization of DC-SIGN. The tyrosine-based motif and the tri-acidic cluster are also involved in internalization. However, the internalization capacities of the homologues of DC-SIGN have not been extensively explored. Based on its expression pattern, mSIGNR1 seems more similar to the homologue of L-SIGN rather than DC-SIGN whereas the expression of mDC-SIGN seems more homologous to DC-SIGN, although they are expressed by different DC subtypes.

DC-SIGN. In contrast to DC-SIGN, L-SIGN and LSECtin are neither expressed by DC in vivo nor by monocyte-derived DC cultured in vitro[40,41], rather both L-SIGN and LSECtin are expressed in liver on liver sinusoidal endothelial cells (LSEC) and in lymph node (Fig. 1.2)[40,41]. Whereas DC-SIGN is expressed on lymph node DC, L-SIGN is found on cells underneath the subcapsular sinus of the lymph node that express the endothelial markers LIVE-1 and CLEVER-1[(44)]. These cells are in direct contact with afferent lymph that not only carries antigens bound to DC, but also transports soluble antigens to the lymph

node. In addition, L-SIGN is expressed by placenta endothelial cells[48].

Both DC-SIGN and L-SIGN contain repeats within the neck domain. In contrast to DC-SIGN that contains 7 complete and one incomplete repeat, the number of repeats of L-SIGN is variable and varies between 3 and 9 (Fig. 1.2)[40]. These neck domains enable multimerization of DC-SIGN and L-SIGN, and both receptors form tetramers[49,50]. Tetramerization of DC-SIGN and L-SIGN may strengthen interactions with ligand by enabling high-avidity binding. Since alleles encoding L-SIGN have variable numbers of repeats in heterozygotic individuals, this may affect proper tetramerization of L-SIGN, and abrogate or modulate ligand binding to L-SIGN. Indeed, Chan *et al.* observed that cells expressing L-SIGN molecules with similar numbers of tandem repeats display a higher ligand binding capacity than cells expressing heterozygous L-SIGN with different numbers of tandem repeats[51].

Through the binding of various pathogens, DC-SIGN also functions as an antigen receptor that internalizes upon binding of antigen, and targets bound antibody to the late endosomal/lysosomal compartment for processing and presentation to T cells[35]. Thus far it is unknown whether L-SIGN has an antigen presenting function.

L-SIGN has considerable homology to DC-SIGN in the cytoplasmic tail and the di-leucine motif, which is essential for internalization through DC-SIGN[52,53], is conserved in L-SIGN, indicating that also L-SIGN functions as an internalization receptor (Fig. 1.2). Indeed, similar to DC-SIGN, L-SIGN mediates internalization of hepatitis C virus (HCV) particles to the endosomal compartment[52].

Binding specificity of DC-SIGN and L-SIGN

The CRD of the SIGN receptors determines their binding specificity. Within the CRD the highly conserved sequences are essential in recognizing mannose-, fucose- or galactose-containing structures. The CRD of DC-SIGN contains an EPN motif, resulting in a high affinity for mannose-type carbohydrates (Fig. 1.2)[54]. DC-SIGN also specifically binds non-sialylated Lewis antigens such as Lewis^a, Lewis^b, Lewis^x, and Lewis^y and this has led to the identification of several pathogens and cellular ligands that are recognized by these receptors[55,56]. DC-SIGN recognizes viruses such as human immunodeficiency virus type 1 (HIV-1) , Dengue virus, human cytomegalovirus, Ebola and HCV, *Mycobacterium tuberculosis*, but also parasites such as *Schistosoma Mansoni* and cellular ligands such as ICAM-2, expressed by endothelial cells, and ICAM-3 present on T cells, through high mannose glycans[43,45,55,57–61].

The interaction of DC-SIGN with cellular ligands such as ICAM-2 and ICAM-3 indicates that DC-SIGN serves as an adhesion receptor to establish cellular interactions between DC and T cells, DC-SIGN/ICAM-3, or DC and endothelial cells, DC-SIGN/ICAM-2[(43,45)]. In addition, DC-SIGN expressed by DC was observed to interact with neutrophils through Lewis^x expressed by Mac-1 and CEACAM1[(56,62)]. Both ligands are upregulated upon neutrophil activation. Strikingly, DC-SIGN specifically interacts with Mac-1 ex-

pressed by neutrophils in contrast to Mac-1 expressed by other cells. This interaction, through the production of TNFα by the neutrophil, results in DC maturation. Upon maturation, the DC produce interleukin (IL)-12, which skews the concomitant immune response to induce a helper T cell (Th) 1 response. Hereby the neutrophil influences the immune response through DC-SIGN.

The highest degree of homology between DC-SIGN and L-SIGN is found within the CRD, indicating that L-SIGN and DC-SIGN have similar ligands (Fig. 1.2). Indeed, L-SIGN, similar to DC-SIGN, recognizes high mannose glycans, and binds high mannose-containing ligands. Similar to DC-SIGN, L-SIGN is able to bind ICAM-3[40], and may establish cellular interactions with ICAM-3-expressing T cells allowing activated T cells to leave the circulation and enter the liver and lymph nodes.

Strikingly, L-SIGN does not bind to the fucose-containing Lewis^x antigen in contrast to DC-SIGN. The difference in the carbohydrate recognition profile of DC-SIGN and L-SIGN has been traced to a single amino acid mutation: Val351 in DC-SIGN to Ser363 in L-SIGN[63,64]. Protein modeling indicates that Val351 forms a hydrophobic pocket that is required to fit Lewis^x into DC-SIGN and to stabilize binding. Lewis^x binding to L-SIGN is disrupted since a hydrophilic serine is present in L-SIGN. This suggests that L-SIGN-expressing endothelial cells of liver and lymph node are not involved in capture of Lewis^x expressed by pathogens nor self structures. Possibly the differential recognition of Lewis^x is involved in the capacity of L-SIGN to discriminate between two Leishmania parasite strains. Whereas DC-SIGN binds both *L. pifanoi* and *L. infantum*, which induce cutaneous and visceral leismaniasis respectively, L-SIGN specifically recognizes only *L. infantum*[65], suggesting that the interaction between L-SIGN expressed by LSEC and *L. infantum* may be involved in the infection of juxtaposing Kupffer cells, the target cells of *L. infantum*.

DC-SIGN and L-SIGN as a pathogen receptor for viruses

Several viruses, such as HIV-1, severe acute respiratory syndrome coronavirus, Dengue virus and Sindbis virus, were found to interact with DC-SIGN and L-SIGN, resulting in infection[48,60,61,66–68]. However the structures involved in the specific interaction with DC-SIGN and L-SIGN have not yet been identified. For the viruses HIV-1, HCV, and Ebola it has been established that L-SIGN and DC-SIGN bind these viruses through recognition of high mannose moieties[40,57,69–71]. Strikingly, some viruses use the binding to DC-SIGN for transport and a more efficient infection of other target cells. This mechanism was first identified for HIV-1. Immature DC-SIGN$^+$ DC are located underneath mucosal epithelium at sites of HIV-1 transmission. After capture of HIV-1 by DC-SIGN on DC, DC may travel to the lymph nodes where CD4$^+$ T cells reside and present HIV-1 for in-*trans* infection of T cells[61]. After internalization through DC-SIGN, HIV-1 is routed to non-lysosomal compartments which allows HIV-1 to escape lysosomal degradation and remain virulent within DC for prolonged times[72,73]. This mechanism may

also be used by other viruses since DC-SIGN also binds and internalizes Dengue virus, human cytomegalovirus, Ebola and HCV for in-*trans* infection of target cells [57–60]. Moreover, these viruses directly infect DC through interactions with DC-SIGN [57,60]. L-SIGN mediates in-*trans* infection of target cells with similar efficiency as DC-SIGN [40,57,59]. In particular, L-SIGN interaction with viruses may be of importance for transmission of hepatotropic viruses such as HCV. Indeed, L-SIGN expressing LSEC have been shown to capture HCV from the blood and transmit the virus to neighboring hepatocytes, in which HCV can replicate [52,74]. Thus, viruses target both DC-SIGN and L-SIGN to facilitate the infection of target cells.

Besides these similarities in the binding to pathogens, DC-SIGN and L-SIGN also display unique binding capacities. Davis *et al.* showed that human cell-derived West Nile virus is specifically recognized by L-SIGN while it was recognized to a much lesser extent by DC-SIGN, indicating that human cell-derived West Nile virus contains a yet unidentified ligand that is specifically bound by L-SIGN but not by DC-SIGN [75].

DC-SIGN and L-SIGN as a pathogen receptor for bacteria and parasites

Although *M. tuberculosis* primarily infects macrophages, it also binds DC through interactions of its cell wall component ManLAM with DC-SIGN [76,77]. Moreover, ManLAM is secreted by *M. tuberculosis*-infected cells, and ManLAM binding to DC-SIGN on DC blocks Toll-like receptor-induced maturation of DC and induces the immunosuppressive cytokine IL-10 [76]. This indicates that binding of ManLAM to DC-SIGN triggers inhibitory signals by which *M. tuberculosis* suppresses immune activation signals through TLR signaling. Recently, probiotic bacteria such as the *Lactobacillus* species *L. reuteri* and *L. casei* were found to also exert immune suppression through engagement of DC-SIGN on DC. However, in contrast to *M. tuberculosis*, these bacteria of the normal gut flora do not inhibit DC maturation, but instruct DC to induce IL-10-producing regulatory T cells that suppress T cell responses [78]. Thus, bacteria use DC-SIGN binding to modulate the immune response.

Non-sialylated Lewis antigens are expressed on LPS of *Helicobacter pylori* and on soluble egg antigens of the parasite *Schistosoma mansoni* and indeed these pathogens bind to DC-SIGN [55,76,79]. Although interaction of Lewis antigen-expressing *H. pylori* with DC-SIGN also results in immune regulation, this is different from the immune modulation exerted by *M. tuberculosis, L. reuteri* and *L. casei*. DC-SIGN binding Lewis$^{x+/y+}$ *H. pylori* induce a mixed Th1/2 response, whereas Lewis^{-} *H. pylori* that do not bind DC-SIGN trigger a Th1 response [80]. Since *H. pylori* can spontaneously switch the expression of the Lewis antigen on LPS, *H. pylori* can evade the recognition by DC-SIGN by down-regulating the Lewis expression. In addition, the phase shift may enable the few Lewis^{+} *H. pylori* in a predominant Lewis^{-} *H. pylori* population to suppress protective Th1 responses, and may provide an explanation for the induction of chronic gastritis by *H. pylori* infection. The parasite *S. mansoni* also triggers Th2 responses, however, it is

presently unknown whether the interaction of Lewis antigens on the parasite with DC-SIGN on DC is involved in the modulation of the evoked immune response towards a Th2 prone response[81].

In conclusion, pathogen binding by DC-SIGN can result in the modulation of the immune response facilitating persistence of the pathogen. Possibly, L-SIGN is also involved in the immune-suppressive mechanisms induced by pathogens.

1.2.2 The murine SIGN homologues

Five murine DC-SIGN homologues have been cloned and have been characterized by several groups[14,82–84]; as a consequence, some of these homologues received different names. The five homologues of DC-SIGN will be addressed here as mDC-SIGN, mSIGNR1 (SIGN-Related), mSIGNR2, mSIGNR3 and mSIGNR4. These five homologues of DC-SIGN are situated on adjacent regions of mouse chromosome 8 A1.2-1.3. Similar to human DC-SIGN, the mDC-SIGN gene is located in close proximity to the CD23 gene, and similar to human L-SIGN, mSIGNR1, 2 and 3 are located near the mDC-SIGN gene.

The five murine homologues display various deviations from the general structure of DC-SIGN (Fig. 1.2). Despite a similarity ranging from 65% to 70% to the amino acids in the CRD region of human DC-SIGN, the murine homologues have a much shorter membrane proximal neck domain compared to their human homologues, with a lesser degree of homology.

Soluble forms of murine DC-SIGN homologues

The murine homologue mSIGNR2 is expressed as a soluble form, since it lacks the cytosolic and the transmembrane domain (Fig. 1.2)[84]. Also, two alternative splicing products of mSIGNR1 were found. One of these products lacked the transmembrane region[84]. The cellular fate and the function of the homologues lacking the transmembrane domain are unknown. These soluble proteins may remain in the cytoplasm and/or may be secreted[85]. The presence of soluble DC-SIGN homologues in the cytoplasm can be useful in the recognition of viral pathogens that have infected the cell, similarly as has been shown for soluble mannose-binding lectin[86]. Secretion of soluble murine homologues of DC-SIGN may act as extracellular antagonists of the membrane-expressed homologues as has been reported for CD23, CD72 and mast cell function-associated antigen[87–89]. Although mSIGNR2 mRNA has been detected in testis and LPS-stimulated B cells none of these soluble molecules have been detected at the protein level yet.

mSIGNR3 and mSIGNR4

mSIGNR3 mRNA has been detected at low levels in spleen and lymph node. At a cellular level, some mSIGNR3 RNA could be detected at low levels in DC, and at even lower levels in T and B cells[84]. However, expression of mSIGNR3 at the protein level remains elusive. Using a soluble IgG-Fc mSIGNR3 chimera, a binding specificity for mannose-, fucose- and N-acetylglucosamine-terminating oligosaccharides was demonstrated[90]. The fucose recognition was demonstrated by the binding to Lewis antigens $Lewis^{a/b}$ and $Lewis^{x/y}$. In addition, mSIGNR3 expressed by transfectants displayed a specificity for both dextran and zymosan[91].

mSIGNR4 mRNA was detected at high levels in testis, similar to mSIGNR2, and at very low levels in spleen. No RNA was detected in either DC, T cells or B cells[84]. In contrast to the other murine SIGN receptors, mSIGNR4 has a QPN motif instead of an EPN motif, indicating that mSIGNR4 preferentially binds galactose-containing carbohydrates (Fig. 1.2)[54,84].

Although there are five murine homologues of DC-SIGN present at DNA level, only mDC-SIGN and mSIGNR1 are expressed by cells of the immune system. Hence, research has focused on mDC-SIGN and mSIGNR1 and little is known so far of the other murine homologues.

mDC-SIGN

All murine homologues were first identified by RT-PCR from murine spleen DC, whereas only mDC-SIGN has been shown to be expressed by DC[83]. By RNA blot analysis, mDC-SIGN mRNA was detected at high levels in spleen, lung, and bone marrow, and at moderate levels in kidney, heart, thymus and lymph node[83,84]. Assessment of the mDC-SIGN RNA at the cellular level showed that mDC-SIGN RNA was abundantly present in DC, at low levels in B cells, but not in T cells[83]. $CD8\alpha^-$ DC express higher levels of mDC-SIGN RNA than $CD8\alpha^+$ DC[83]. Similar to human DC-SIGN on DC, the expression of mDC-SIGN is down-regulated upon DC activation and maturation[83]. Interestingly, mDC-SIGN is expressed on plasmacytoid preDC that differentiate into $CD8^+CD205^-$ DC upon activation with microbial stimulus (Fig. 1.2)[92]. These data suggest that, although the DC subsets are different, mDC-SIGN being DC-specific most closely resembles DC-SIGN.

Recent studies have shown that mDC-SIGN is not equipped to bind to multivalent pathogen ligands such as Ebolavirus glycoprotein and HIV-1 gp120 (our unpublished observations)[93]. Using murine and human DC-SIGN chimeras it was shown that both the neck domain, as well as the CRD, prevents interaction of mDC-SIGN with multivalent ligands. Therefore, despite the similarity in expression and the homology of amino acids in the CRD, mDC-SIGN does not appear to be suited to function as a model for further investigations into the in vivo function of DC-SIGN. However, mDC-SIGN can be used as a tool to specifically target the $CD8^-$ DC population[94].

mSIGNR1

The most extensively studied of the 5 murine homologues of DC-SIGN is mSIGNR1. mSIGNR1 mRNA has been demonstrated at high levels in lymph nodes, moderate levels in both spleen and testis, but little or no expression in other tissues [13,84]. Hardly any or no mSIGNR1 expression was found on DC, alveolar macrophages, B cells and T cells [13,95]. Identification of mSIGNR1 as the antigen for the MZM-specific antibody ERTR-9, led to a more detailed investigation of its expression pattern [14]. mSIGNR1 protein was found to be expressed in lymph node, spleen and liver. In addition, Taylor *et al.* demonstrated that mSIGNR1 is expressed on resident peritoneal macrophages [95], whereas thioglycollate elicited macrophages do not express mSIGNR1 [96]. Besides mSIGNR1, resident peritoneal macrophages also express the C-type lectin Dectin-1. Both mSIGNR1 and Dectin-1 are involved in the capture of the yeast component zymosan [95]. In the lymph node, mSIGNR1 is expressed on the medullary and subcapsular macrophages [14] (Fig. 1.2). In humans, L-SIGN is expressed by cells located underneath the subcapsular sinus of the lymph node [44]. Possibly, both L-SIGN and mSIGNR1 are involved in the capture of antigen from circulating lymph.

In the liver the expression of mSIGNR1 is similar to L-SIGN as they are both expressed by liver LSEC [14], which are specialized endothelial cells that are localized between the liver sinusoids and hepatocytes. Here, these fenestrated endothelial cells, which lack a basal membrane, function as a barrier to protect the hepatocytes from blood-borne harmful components while allowing for exchange of nutrients [97]. These specialized endothelial cells with antigen presenting cell characteristics internalize components via receptor-mediated endocytosis and are able to transcytose these agents across the created barrier. Lohse *et al.* have described that LSEC are able to present antigen to, and prime, naive $CD4^+$ T cells similar to DC [98]. Strikingly, LSEC induce immune tolerance [99].

Recently it has been described that murine LSEC are able to cross-present food antigens on their MHC I molecules within 2 hours after ingestion [100]. Similar to $CD4^+$ T cells, the functional outcome of $CD8^+$ T cell stimulation by cross-presenting LSEC is tolerance [101]. Therefore it seems that LSEC are a liver-specific antigen presenting cell population that is organ resident and induces immune tolerance. LSEC are known for their highly efficient receptor-mediated endocytosis of macromolecules. The expression of pattern recognition receptors by LSEC facilitates the internalization of substantial amounts of molecules for MHC-restricted presentation to T cells. Pattern recognition receptors expressed by LSEC are the mannose receptor and scavenger receptors. However, these receptors are expressed quite ubiquitously. In contrast, the C-type lectin L-SIGN and its murine homologue mSIGNR1 are specifically expressed by LSEC and not by other liver resident immune cells such as Kupffer cells [14,40,48,84]. The function of these C-type lectins during homeostasis is currently unclear. Future experiments on the function of mSIGNR1 expressed by LSEC will unravel its in vivo function and may serve as a model for the in vivo function of L-SIGN.

1.3 The in vivo function of the SIGN molecules

Although mSIGNR1 is not expressed by DC but by specific macrophage subsets, its function has been studied in more detail than mDC-SIGN. mSIGNR1 has a binding activity for hICAM-2 and hICAM-3 similar to human DC-SIGN [14,82]. Thus, mSIGNR1 can function as an adhesion receptor. However, mice do not express an ICAM-3 homologue and therefore binding of mSIGNR1 to ICAM-3 is not physiologically relevant. mSIGNR1 also interacts with murine ICAM-2, which is widely expressed on murine lymphocytes, and could therefore mediate contact between mSIGNR1-positive cells and leukocytes in mice [102].

Besides being a gateway for migrating lymphocytes, the splenic marginal zone is also important as a defense against pathogens (reviewed by Kraal [103]). Because of their position adjacent to the marginal sinuses, MZM are among the first cells to interact with blood-borne antigens and are presumed to have a critical role in host defense against bacterial pathogens. The fact that mSIGNR1 is abundantly expressed by MZM [13,14] hints to a function as a pathogen recognition receptor. Indeed, mSIGNR1 is able to capture the model polysaccharide antigen dextran, and mannan, the major constituent of the yeast-derived particle zymosan directly from blood [13,14]. Hence, mSIGNR1 functions as a pathogen receptor contributing to the filter function of the spleen. The spleen is known to be pivotal for the clearance of blood-borne pathogens such as *Streptococcus pneumoniae*, lymphocytic choriomeningitis virus, ectromelia virus and Sindbis virus [104–108]. It remains to be seen whether mSIGNR1 merely scavenges the pathogens from the blood or whether mSIGNR1 is also involved in the presentation of these captures antigens to closely situated marginal zone B cells (Fig. 1.1). The situation of the MZM and MZB, which reside in close contact to each other, would allow for such interactions, resulting in a role for mSIGNR1 in the TI immune response.

1.4 Outline of this thesis

The binding characteristics of murine SIGNR1 was investigated and compared to both human homologues; DC-SIGN and L-SIGN (Chapter 2) [109]. Both DC-SIGN and mSIGNR1 share a preference for multi-mannose coupled to the lipoarabinomannan (LAM) structure of mannose-capped LAM (ManLAM) , a capsular component of *Mycobacterium tuberculosis* (Chapter 3) [110]. To investigate the in vivo consequences of the recognition of *M. tuberculosis* by mSIGNR1, mSIGNR1-deficient mice were studied in an infection model with *M. tuberculosis* in Chapter 4. Although the immunosuppressive cytokine IL-10 was induced upon in vitro stimulation of peritoneal macrophages with ManLAM, and enhanced early systemic immune response in the mSIGNR1-deficient mice was observed, no differences in pathology were apparent. Our results indicate that mSIGNR1 does not play a crucial role in *M. tuberculosis* pathology since mSIGNR1 is not expressed in the lungs but at sites remote from the site of infection. Therefore we investigated the in vivo

function of mSIGNR1 during infection with a pathogen known to depend on the spleen for an effective immune response; *S. pneumoniae* [111].

First the interaction of DC-SIGN with *Streptococcus pneumoniae* was investigated (Chapter 5) [112]. From a panel of *S. pneumoniae* serotypes we demonstrated that DC-SIGN specifically interacted with 2 serotypes; serotype 3 and 14 and not with serotype 19. The binding of *S. pneumoniae* serotype 14 was mediated through the capsular polysaccharide. For serotype 3 the ligand bound by DC-SIGN remains unidentified. Similar to DC-SIGN, mSIGNR1 recognizes *S. pneumoniae*. This is of specific interest since the spleen plays an important role in the immune defense against encapsulated Gram-positive *S. pneumoniae*; this bacterium is notorious for its overwhelming post-splenectomy infections and has been observed to target the marginal zone of the spleen where the mSIGNR1 expressing marginal zone macrophages reside (Fig. 1.1). We therefore set out to investigate in Chapter 6 the in vivo function of mSIGNR1 during infection with *S. pneumoniae* [113]. Upon infection with *S. pneumoniae*, we observed a striking difference in pathology between wild type and mSIGNR1-deficient mice. The mSIGNR1-deficient mice displayed more severe pathology with a larger influx of cells and a larger outgrowth of the bacteria in various organs. In addition, the amount of early IgM antibodies reactive with PC, expressed by *S. pneumoniae*, was analyzed and was very low in mSIGNR1-deficient mice compared to wild-type mice, indicating a possible role of mSIGNR1 in the production of early anti-PC antibodies. Likely, these early anti-PC antibodies are produced by marginal zone B cells, which reside in close contact with the mSIGNR1-expressing marginal zone macrophages of the spleen (Fig. 1.1). In Chapter 7 we investigated the interaction between mSIGNR1 and marginal zone B cells more closely. Indeed we observed that mSIGNR1 is able to interact with marginal zone B cells. Although a decrease in the amount of marginal zone B cells was observed in mSIGNR1-deficient mice despite a normal location of this specific cell population, the functional consequences of this interaction are not fully understood yet.

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Chapter 2

Carbohydrate specificities of the murine DC-SIGN homologue mSIGNR1

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Abstract

C-type lectins are important receptors expressed by antigen presenting cells that are involved in cellular communications as well as in pathogen uptake. An important C-type lectin family is represented by DC-SIGN and its homologues in human and mouse. Here we have investigated the carbohydrate specificity of cellular mSIGNR1 and compared it with DC-SIGN and L-SIGN. mSIGNR1 has a similar specificty as human DC-SIGN for high mannose-containing ligands present on both cellular and pathogen ligands. However, the SIGN molecules differ in their recognition of Lewis antigens; mSIGNR1 interacts not only with Lewis$^{x/y}$ and Lewis$^{a/b}$ antigens similar to DC-SIGN, but also with sialylated Lewis^{x}, a ligand for selectins. The differential recognition of Lewis antigens suggests differences between mSIGNR1 and DC-SIGN in the recognition of cellular ligands and pathogens that express Lewis epitopes.

2.1 Introduction

Antigen presenting cells such as macrophages and dendritic cells (DC) express C-type lectins that are involved in pathogen capture, processing and antigen presentation to induce immune responses against these pathogens [1]. However, it is becoming clear that several lethal pathogens have evolved to subvert the function of some C-type lectins to escape immune surveillance [2]. An important C-type lectin family is represented by DC-SIGN and its homologues in both human and mouse.

The "SIGN" family of C-type lectin receptors (CLR) consists of DC-SIGN and L-SIGN in human and the five murine homologues mDC-SIGN, mSIGNR1 to mSIGNR4. Human DC-SIGN is expressed by DC [2,3], whereas L-SIGN is expressed on specialized liver and lymph node endothelial cells that possess antigen presentation capacity [4–6]. In mouse, only mDC-SIGN is expressed by DC [7], whereas the mSIGNR1-4 molecules display a differential tissue distribution [8]. The most extensively studied of these murine homologues is mSIGNR1, which is expressed on marginal zone macrophages (MZM) [8,9] and peritoneal macrophages [10].

DC-SIGN has been demonstrated to interact with various viruses such as HIV-1 [11], human cytomegalovirus [12], and Dengue virus [13] and microbes including *M. tuberculosis* [14,15] and *Leishmania* parasites [16]. DC-SIGN has been shown to mediate internalization of ligands for antigen presentation [17]. Strikingly, HIV-1 exploits this feature of DC-SIGN to gain access to CD4^{+} T cells that are the primary target cells of infection [11]. Similarly, other viruses have been shown to use DC-SIGN to enhance viral transmission to target cells [2], whereas *M. tuberculosis* and *Helicobacter pylori* target DC-SIGN to modulate DC function [14,18]. Thus, several pathogens target DC-SIGN to escape immune surveillance. Identification of the carbohydrate specificity of DC-SIGN for high mannose glycans and non-sialylated fucose-containing Lewis (Le) antigens, such as Le^{a}, Le^{b}, Le^{x} and Le^{y} [19–21],

has led to the identification of both pathogens and self-antigens that are recognized by this receptor. The high degree of homology between the carbohydrate recognition domains of DC-SIGN and L-SIGN is reflected by their similar ligand specificity. L-SIGN, similar to DC-SIGN, recognizes high mannose glycans, and binds high mannose-containing ligands such as HIV-1 and HCV[4,22]. However, L-SIGN does not bind to the fucose-containing Lewis antigens in contrast to DC-SIGN[23].

The carbohydrate specificity of mSIGNR1 demonstrated specificity for mannose- and fucose-terminating oligosaccharides[24,25]. By using a soluble recombinant chimera of mSIGNR1 fused to an Fc domain, Galustian *et al.* demonstrated that mSIGNR1 binds to the Lewis antigens $Le^{x/y}$ and $Le^{a/b}$, similar to DC-SIGN[24]. Although the use of Fc chimeras provides information about the binding characteristics of C-type lectins, studies into their binding specificity should also be performed with the C-type lectins expressed on cells due to their multimerization and possible clustering, which is crucial to the carbohydrate binding specificity. Indeed, mSIGNR1-Fc did not interact with the polysaccharide dextran, whereas cellular-expressed mSIGNR1 interacts with dextran as demonstrated by several groups[8,9,24].

Here, we have investigated and compared the carbohydrate binding specificity of cellular mSIGNR1 with its human homologues DC-SIGN and L-SIGN. DC-SIGN, L-SIGN and mSIGNR1 have a similar specificity for mannose-containing carbohydrates present on both cellular and pathogen ligands. However, the three receptors differ in their recognition of fucose-containing Lewis antigens. mSIGNR1 interacts not only with $Le^{x/y}$ and $Le^{a/b}$ antigens similar to DC-SIGN, but also with sialylated Le^{x}. The differential recognition of Lewis antigens suggests differences between mSIGNR1 and DC-SIGN in the interaction with Lewis antigen-containing cellular and pathogen ligands.

2.2 Materials and Methods

Antibodies, reagents and cells

The following antibodies were used: ERTR-9 (anti-mSIGNR1)[9], AZN-D1 (anti-DC-SIGN), AZN-D2 (anti-L-SIGN/DC-SIGN)[3], Raji-1 (previously referred to as THP-1 cells [26]) transfectants expressing wild-type DC-SIGN, L-SIGN or mSIGNR1 were generated by transfection with 10 μg pRc/CMV-DC-SIGN plasmid by electroporation as previously described[3,4,9]. ManLAM was obtained from J.T. Belisle, the TB Vaccine Testing and Reseach Materials Contract NIAID N01-AI-40091, Colorado State University, CO. Purified lipopolysaccharide (LPS) from *H. pylori* was obtained from M. Monteiro (National Research Council, Ottawa, Canada).

Fluorescent bead adhesion assay

Carboxylate-modified TransFluorSpheres (488/645 nm, 1.0 μm; Molecular Probes, Eugene, OR) were coated with HIV-1 gp120 and ICAM-2 as described[11]. Mannan was covalently coupled to the beads according to the manufacturers protocol. Streptavidin was covalently coupled to the beads as described and streptavidin-coated beads were incubated with biotinylated PAA-linked glycoconjugates (50 pmol; Syntesome, Munich, Germany). The fluorescent bead adhesion assay was performed as described[27]. Cells were preincubated for 30 minutes at 37°C with inhibitors (mannan, 1 mg/ml or EGTA, 10 mM). Next, ligand-coated fluorescent beads (20 beads/cell) were added to the cells for 45 minutes at 37°C, washed and analyzed by flow cytometry (FACScan, Becton Dickinson, Oxnard, CA), by measuring the percentage of cells that had bound fluorescent beads.

2.3 Results

In order to investigate the interaction of mSIGNR1 with different carbohydrates and to compare its ligand specificity with the human homologues DC-SIGN and L-SIGN, we stably transfected Raji-1 cells with these receptors (Fig. 2.1A). The cells express high levels of the different C-type lectins (Fig. 2.1A). The expression level of mSIGNR1 was measured with a different antibody (ERTR-9) than the human homologues (AZN-D2) and due to differences in affinity the expression levels cannot be compared to each other. Recently, we have demonstrated that human DC-SIGN and L-SIGN and murine SIGNR1 interact with mycobacteria through mannose-capped lipoarabinomannan (ManLAM)[25]. Both homologues have the highest affinity for the $(man)_3$-ara structure present on ManLAM, similar to DC-SIGN[25]. Therefore we compared the interaction of the three cell-lines with ManLAM. The binding activity of the transfectant expressing mSIGNR1 to ManLAM is similar to that observed for the two human homologues DC-SIGN and L-SIGN (Fig. 2.1B), indicating that the different transfectants have comparable expression levels of the three C-type lectins and can be used to compare their carbohydrate specificity.

Next, we investigated the interaction with the poly mannose-containing polysaccharide mannan. This polysaccharide has been extensively used as an inhibitor of DC-SIGN and other C-type lectins that have specificity for high mannose structures because this ligand is multivalent and mannose-specific C-type lectins have a high affinity for this polysaccharide[28]. Here we have coated fluorescent beads with mannan through chemical cross-linking in order to investigate the interaction with mSIGNR1. As demonstrated, DC-SIGN and its homologues bind strongly to the poly mannose-containing carbohydrate. The interaction is specific since the mock transfectant does not interact with mannan (Fig. 2.1B) and blocking studies demonstrate that this interaction can be inhibited by the calcium chelator EGTA (data not shown). As reported earlier, DC-SIGN also binds to less complex mannose-containing glycoconjugates i.e. mannose and

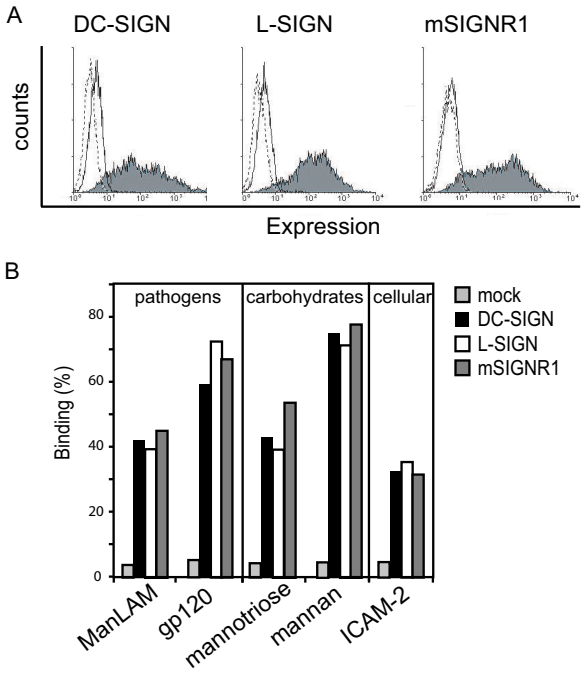

Figure 2.1: Cellular mSIGNR1 interacts with high mannose-containing polysaccharides
(A) Raji-1 transfectants express high levels of DC-SIGN, L-SIGN and mSIGNR1. Open histograms represent the isotype controls, dotted lines represent mock transfectants and filled histograms indicate specific antibody staining. (B) Cellular mSIGNR1 interacts specifically with high mannose epitopes present on polysaccharides and glycoproteins. Binding of the SIGN transfectants to the different ligands was determined using the fluorescent bead adhesion assay. Standard deviation for the fluorescent bead adhesion assay was $< 5\%$. One representative experiment out of three is shown.

$\alpha1\rightarrow3,\alpha1\rightarrow6$mannotriose [19,20]. We observed a similar binding of mSIGNR1 to $\alpha1\rightarrow3,\alpha1\rightarrow6$mannotriose-coated beads, as was observed with DC-SIGN and L-SIGN. These data support previous studies demonstrating that mSIGNR1 has a similar binding specificity for both high mannose and less complex mannose-containing carbohydrate structures. These data suggest that mSIGNR1 interacts similarly to cellular ligands and pathogens containing these mannose structures. Indeed, mSIGNR1 interacts with high mannose-containing ligands of DC-SIGN, such as ICAM-2/-3 [9], HIV-1 gp120 [29], zymosan [30] and mycobacteria [10,25] (Fig. 2.1B).

Although L-SIGN and DC-SIGN recognize high mannose glycans, L-SIGN does not bind to fucose-containing Le^x antigens in contrast to DC-SIGN [21]. Using Fc chimera of mSIGNR1, Galustian *et al.* demonstrated that mSIGNR1, similar to DC-SIGN, binds

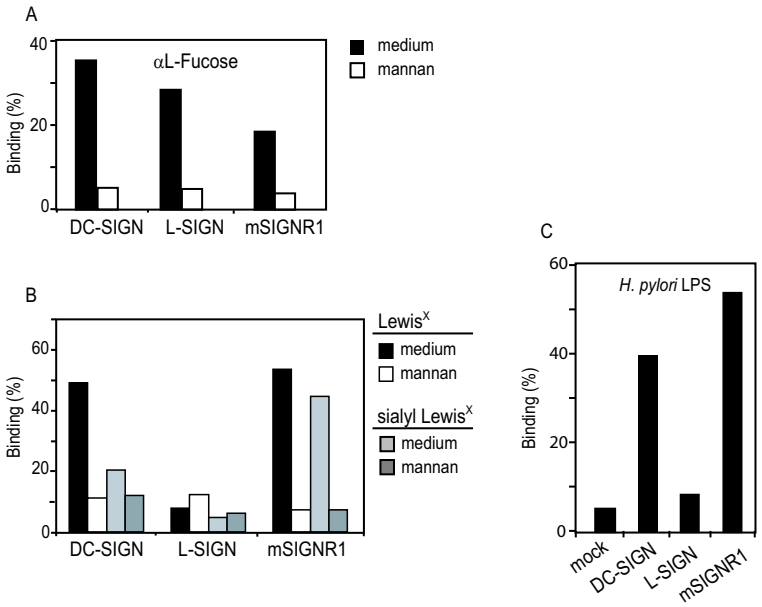

Figure 2.2: Cellular mSIGNR1 interacts with sialylated Le^x, in contrast to its human homologues

The three DC-SIGN homologues differ in their recognition of fucose-containing carbohydrates; all three C-type lectins bind fucose (A), whereas DC-SIGN and mSIGNR1, in contrast to L-SIGN, bind Le^x (B). The recognition of Le^x-rich LPS from *H. pylori* by DC-SIGN and mSIGNR1 is determined by their carbohydrate specificity (C). Binding of the SIGN transfectants to the different ligands was determined using the fluorescent bead adhesion assay. Specificity was determined by measuring binding in the presence of mannan. Standard deviation for the fluorescent bead adhesion assay was $< 5\%$. One representative experiment out of three is shown.

to the Lewis antigens Le$^{x/y}$and Le$^{a/b}$ [24]. Here we have investigated the interaction of cellular mSIGNR1 with the Lewis antigens. Similar to DC-SIGN and L-SIGN, cellular mSIGNR1 interacts with αL-fucose through its C-type lectin domain, since the binding is inhibited by both mannan (Fig. 2.2A) and EGTA (data not shown). A screening of the different Lewis antigens coated on fluorescent beads demonstrate that cellular mSIGNR1 interacts to Lewis blood group antigens (Le^x, Le^y, Le^a, Le^b) that contain fucose residues in different anomeric linkages, similar to DC-SIGN (Table 2.1). Moreover, sulfation of these Lewis antigens does not abrogate binding (Table 2.1). Strikingly, mSIGNR1 interacts with sialylated Le^x, an L-, E- and P-selectin ligand, whereas sialylation of Le^x completely abrogates the recognition by DC-SIGN (Fig. 2.2B; Table 2.1). A more detailed analysis demonstrates that the interaction of mSIGNR1 with sialyl-Le^x is mediated by the C-type lectin domain since it can be inhibited by both mannan and EGTA (Fig. 2.2 B and data

not shown).

These data demonstrate that although mSIGNR1 has a similar specificity for high mannose-containing ligands as DC-SIGN and L-SIGN, the SIGN family differs in its recognition of Lewis antigens; mSIGNR1, in contrast to DC-SIGN and L-SIGN, interacts with sialylated Le^x, whereas L-SIGN does not bind Le^x. Lewis antigens are expressed on various cells and pathogens and our data indicate that mSIGNR1 may be an important receptor on macrophages to facilitate the interaction with sialyl-Le^x expressed by endothelial cells and T cells, as well as with Lewis antigen-expressing pathogens such as the gram-negative bacterium *H. pylori*, which induces peptic ulcers and gastric carcinoma [31], and the worm parasite *Schistosoma mansoni* (the causal agent of schistosomiasis) [32]. Indeed, mSIGNR1, similar to DC-SIGN, interacts with LPS from *H. pylori* that is rich in Le^x, whereas L-SIGN does not interact with this Le^x-rich LPS (Fig. 2.2C).

2.4 Discussion

Here we have investigated the carbohydrate specificity of cellular mSIGNR1, since multimerization and clustering on the cell surface is essential for ligand recognition and interactions. We demonstrate that although mSIGNR1 has a similar binding specificity for high mannose-containing ligands as the human homologues DC-SIGN and L-SIGN, it differs in its interaction with Lewis antigens. Both DC-SIGN and mSIGNR1 interact with Le^x in contrast to L-SIGN, but only mSIGNR1 interacts with sialylated Le^x. These data suggest that mSIGNR1 may share its ligands with P-, L- and E-selectin, which are able to interact with sialylated Lewis antigens. Both DC-SIGN and L-SIGN recognize high mannose glycans as well as fucose-containing Lewis antigens.

L-SIGN does not bind to the fucose-containing Le^x antigens in contrast to DC-SIGN. The difference in the carbohydrate recognition profile of DC-SIGN and L-SIGN has been traced to a single amino acid mutation: Val351 in DC-SIGN to Ser363 in L-SIGN [23,33]. Protein modelling indicates that Val351 forms a hydrophobic pocket that is required to fit Le^x into DC-SIGN and to stabilize binding. Since a hydrophilic serine is present in L-SIGN, Le^x binding to L-SIGN is disrupted. mSIGNR1 contains a hydrophobic isoleucine at the corresponding site (Ile289) indicating that this C-type lectin is also able to form a hydrophobic pocket for Le^x binding.

Galustian *et al.* demonstrated using a Fc chimera of mSIGNR1 that mSIGNR1 has specificity for mannose- as well as fucose-terminating oligosaccharides, as demonstrated by the ability to interact with high mannose N-glycans and proteins containing N-linked glycans such as invertase and soybean agglutinin [24]. The fucose recognition was demonstrated by binding of mSIGNR1-Fc chimera with Lewis antigens $Le^{x/y}$ and $Le^{a/b}$. We have used fluorescent beads coated with the Lewis antigen and demonstrate that cellular mSIGNR1 is indeed able to interact with Lewis antigens as well as their sulphated forms (Table 2.1). In contrast to Galustian *et al.* [24], we observe a strong binding of sialylated Le^x to mSIGNR1 suggesting that possible multimerization and clustering of cellular

Ligand*	DC-SIGN	L-SIGN	mSIGNR1
Le^x	+	-	+
Le^y	+	+	+
Le^a	+	+	+
Le^b	+	+	+
Sialyl Le^x	-	-	+
Sialyl Le^a	-	-	+/-
Sulfo Le^x	+	+/-	+
Sulfo Le^a	+	+	+
αL-fucose	+	+	+

Table 2.1: Binding specificity of DC-SIGN, L-SIGN and mSIGNR1 for Lewis antigens
* Binding was determined by coating the carbohydrates on fluorescent beads and performing the bead adhesion assay with Raji-1 transfectants expressing the C-type lectins (0-10% = -, 10-20% = +/-, 20-50% = +).

mSIGNR1 enhances the interaction/recognition of these structures. Moreover, the antigens were coated on beads and are presented to the receptor as a multivalent structure thereby further increasing the interaction. Thus, although the use of Fc chimeras provides information about the binding characteristics of the CLR, studies into their binding specificity should also be performed with the CLR expressed on cells due to their multimerization and possible clustering. Low affinity interactions may not be detected by Fc chimeras.

Knowledge about the carbohydrate specificity of mSIGNR1 will help identify potential ligands of mSIGNR1 and the in vivo role of this receptor. Lewis antigens are expressed on various cells, especially sialylated Lewis antigens that act as ligands for P-, E- and L-selectins. These selectins play a major role in migratory processes of monocytes, neutrophils and lymphocytes across endothelial cells and mSIGNR1 may be involved in the migration of macrophages, since this C-type lectin is expressed by macrophages. Similarly, DC-SIGN has been demonstrated to function as an adhesion receptor on DC that mediates both rolling on and migration across endothelial cells [34]. The expression of mSIGNR1 on the MZM that are in close contact with blood suggests that this receptor may also be involved in migration of cells from blood into the spleen. Interestingly, lymphocyte migration from blood to the white pulp may be dependent on C-type lectin interactions since polysaccharides such as mannan, a ligand for mSIGNR1 (Fig. 2.1) inhibit this process [35–37].

Besides being a gateway for migrating lymphocytes, the splenic marginal zone is also important as a defense against pathogens. Because of their position adjacent to the marginal sinuses, MZM are among the first cells to interact with blood-borne antigens and are presumed to have a critical role in host defense against bacterial pathogens [38]. The

fact that mSIGNR1 is abundantly expressed by MZM [8,9] hints to a function as a pathogen recognition receptor. High mannose structures are expressed by various pathogens, including viral glycoproteins as well as mycobacteria [10,14,25]. The Lewis-specificity of mSIGNR1 may be important for its interaction with pathogens such as *H. Pylori* (Fig. 2.2C) and certain *Klebsiella pneumoniae* strains through Le^x-containing LPS structures [18], and parasites such as *S. mansoni* as has been demonstrated for DC-SIGN [19,21]. Further studies will demonstrate the role of mSIGNR1 in both infections and adhesion events.

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Chapter 3

Identification of the mycobacterial carbohydrate structure that binds the C-type lectins DC-SIGN, L-SIGN and mSIGNR1

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Abstract

Mycobacterium tuberculosis represents a worldwide health risk, and although macrophages are primarily infected, dendritic cells (DC) are important in inducing cellular immune responses against *M. tuberculosis*. Recent studies have demonstrated that *M. tuberculosis* targets the DC-specific C-type lectin DC-SIGN to inhibit the immunostimulatory function of DC through the interaction of the mycobacterial mannosylated lipoarabinomannan (ManLAM) to DC-SIGN, which prevents DC maturation and induces the immunosuppressive cytokine IL-10. This may contribute to survival and persistence of *M. tuberculosis*. Here we have identified the specific pathogen-derived carbohydrate structure on ManLAM that is recognized by DC-SIGN. We have synthesized the mannose cap oligosaccharides man-ara, $(man)_2$-ara and $(man)_3$-ara, and demonstrate that these neoglycoconjugates are specifically bound by DC-SIGN. Moreover, we demonstrate that the human and murine DC-SIGN homologue L-SIGN and SIGNR1, respectively, also interact with mycobacteria through ManLAM. Both homologues have the highest affinity for the $(man)_3$-ara structure, similar to DC-SIGN. This study provides information about the specific carbohydrate structures on pathogens that are recognized by DC-SIGN, and may provide strategies to develop vaccines against these pathogens. Moreover, the identification of mSIGNR1 as a receptor for ManLAM will enable in vivo studies to investigate the role of DC-SIGN in *M. tuberculosis* pathogenesis.

3.1 Introduction

Tuberculosis has been a major worldwide cause of death for centuries. One-third of the world population is infected with *Mycobacterium tuberculosis*, which causes 2 million deaths per year. Although macrophages, and not dendritic cells (DC), are the primary targets for infection by mycobacteria, DC are important for the cellular immune response and recent data demonstrate that DC function is modulated by *M. tuberculosis* [1,2], which may account for pathogen survival and persistence.

Immature DC express several receptors that have been demonstrated to act as receptors on macrophages, such as the mannose receptor (MR), CD11b and CD11c [3,4]. However, recent data demonstrate that DC-specific C-type lectin DC-SIGN is the major receptor for *M. tuberculosis* on DC [1,5]. DC-SIGN interacts with *M. tuberculosis* through its cell wall component mannosylated lipoarabinomannan (ManLAM) [1]. Strikingly, *M. tuberculosis* modulates the immune function of DC through the interaction of DC with ManLAM [1,2]. ManLAM interacts with DC-SIGN on immature DC thereby blocking LPS-induced maturation and inducing the anti-inflammatory cytokine interleukin (IL)-10[(1)].

Moreover, Nigou *et al.* demonstrated that ManLAM binding to immature DC via another C-type lectin, MR, blocks IL-12 production [2]. These data suggest that ManLAM can interfere with Toll-like receptor (TLR)4 signaling on DC by binding to the C-type lectin

DC-SIGN, since DC maturation by LPS is mediated through TLR4[6]. The cell wall component ManLAM is also secreted in vivo by macrophages infected with *M. tuberculosis*[7,8], indicating that mycobacteria may secrete ManLAM to interfere with bystander DC. Both DC and macrophages express various C-type lectins that capture and internalize pathogens, and recent studies suggest that C-type lectins may also influence the immune response through signaling cross talk especially with TLR[2,9,10]. Recent studies suggest that pathogens may exploit this cross talk to interfere with TLR signaling, thereby modulating immune responses[9–11].

DC-SIGN functions as an adhesion receptor on immature DC that mediates DC interactions with T cells and endothelial cells[12,13]. However, it is becoming clear that several pathogens target DC-SIGN to escape immunosurveillance[11]. DC-SIGN plays a key role in the dissemination of HIV-1 by DC through HIV-1 gp120 binding[14]. Resident mucosal DC may capture HIV-1 through DC-SIGN, and DC-SIGN does not mediate infection of DC but protects the virus during migration to the lymphoid tissues, where DC-SIGN facilitates the transmission of HIV-1 to T cells[14]. Clinical strategies targeting C-type lectins such as DC-SIGN could succeed in combating *M. tuberculosis* infections by shifting the precarious balance between immune activation and suppression to favor the elimination of mycobacteria. More knowledge about the specific pathogen structures recognized by DC-SIGN will be very useful to design strategies to prevent pathogen interactions.

C-type lectins have a high affinity for carbohydrate structures[15]. Although the carbohydrate specificity of DC-SIGN has been intensively characterized[15–19], the specific carbohydrate structures, present on pathogens, that are recognized by DC-SIGN are not well characterized. Here, we have identified the specific carbohydrate structures on ManLAM that are recognized by DC-SIGN. Moreover, we have investigated the interaction of ManLAM with the human DC-SIGN homologue L-SIGN and the murine homologue SIGNR1. Both L-SIGN and mSIGNR1 specifically interact with ManLAM and rapidly internalize ManLAM similar to DC-SIGN.

Therefore, identification of the specific carbohydrate structure recognized by DC-SIGN and its homologues will provide strategies to combat *M. tuberculosis* interactions with these receptors. Moreover, similar binding characteristics of murine SIGNR1 and human DC-SIGN will enable in vivo studies into the role of DC-SIGN in *M. tuberculosis* infections and the efficacy of vaccines.

3.2 Materials and Methods

Antibodies

The following antibodies were used: ERTR-9 (anti-SIGNR1)[20], AZN-D1 (anti-DC-SIGN), AZN-D2 (anti-L-SIGN/DC-SIGN)[12], F30.5 (anti-LAM; A. Kolk (Royal Tropical Institute, Amsterdam)).

Cells

THP-1 and K562 transfectants expressing wild-type DC-SIGN, L-SIGN or mSIGNR1 were generated by transfection with 10 μg pRc/CMV-DC-SIGN plasmid by electroporation as previously described[14,20–22].

Synthesis of LAM oligosaccharides

The synthesis of LAM oligosaccharides has been described previously[23]. In brief, glycosylation reactions were carried out with thioglycoside donors in dichloromethane using activation by N-iodosuccinimide and silver triflate. The oligosaccharides carry an octylamine spacer at the reducing terminus to allow coupling to carriers to produce polyvalent antigens. Various structures have been synthesized: 8-Aminooctyl-5-*O*-{3,5-di-*O*-(2-*O*-[β-D-arabinofuranosyl]-α-D-arabinofuranosyl)-α-D-arabinofuranosyl}-α-D-arabinofuranoside, in short $(\text{ara})_6$, 8-Aminooctyl-β-D-arabinofuranoside, in short ara; 8-Aminooctyl-5-*O*-(α-D-mannopyranosyl)-β-D-arabinofuranoside, in short man-ara; 8-Aminooctyl-5-*O*-((2-*O*-α-D-mannopyranosyl)-α-D-mannopyranosyl)-β-D-arabinofuranoside, in short $(\text{man})_2$-ara; 8-Aminooctyl-5-*O*-(2-*O*-(2-*O*-(α-D-mannopyranosyl)-α-D-mannopyranosyl)-α-D-mannopyranosyl)-β-D-arabinofuranoside, in short $(\text{man})_3$-ara (Fig. 3.1A and B).

Synthesis of biotin labeled polyacrylamide (PAA-biotin) neoglycoconjugates

Biotin-labeled polymeric glycoconjugates were synthesized by coupling of aminooctyl glycosides and biotin-$\text{NH}(\text{CH}_2)_6\text{NH}_2$ with poly(4-nitrophenyl acrylate) as described by Bovin *et al.*[24]. In brief, biotin-labeled (5%) arabinose (20%) polyacrylamide (PAA) conjugate was prepared. A solution of poly(4-nitrophenyl acrylate) (20 mg/ml in DMF, 242 μl, 25 μM on monomer unit), containing 5 mol% of biotin-$\text{NH}(\text{CH}_2)_6\text{NH}_2$, and Et_3N (7 μl) was added to a solution of Ara-$\text{O}(\text{CH}_2)_8\text{NH}_2$ (1.4 mg, 5 μM) in DMSO (100 μl). The mixture was kept 24 hours at 40°C, then 35 μl of ethanolamine was added and the mixture was kept 15 hours at room temperature. Conjugation was monitored by thin layer chromatography. The resulting conjugate was isolated on a 1.5 $\text{cm}^2\times$25 cm Sephadex LH-20 column in a $\text{MeCN}/\text{H}_2\text{O}$ (1:1, v/v) mixture. The lyophilised yield was 3.9 mg (90%). The average degree of polymerization of the (non-substituted) poly(*N*-2-hydroxyethylacrylamide) carrier was estimated by gel-permeation chromatography. The apparent molecular weight was 30 kDa, equivalent to a polymerization degree of $\sim$260. From our earlier studies we know that the reaction of PAA with amino-linked oligosaccharide or biotin runs to completion, and hence the degree of substitution was calculated from the input. It was thus calculated that the PAA neoglycoconjugates contain 50 oligosaccharide and 10 biotin residues per polymeric chain.

Fluorescent bead adhesion assay

Carboxylate-modified TransFluorSpheres (488/645 nm, 1.0 μm; Molecular Probes, Eugene, OR) were coated with the glycolipid forms of LAM as follows. Streptavidin-coated beads[25] were incubated with biotinylated $F(ab)_2$ fragment goat anti-mouse IgG (6 μg/ml; Jackson Immunoresearch) followed by an overnight incubation with mouse-anti-LAM antibody (F30.5) at 4°C. The beads were washed and incubated with 250 ng/ml purified glycolipid LAM overnight at 4°C. The biotin-labeled PAA neoglycoconjugates (0.5 μg/ml) were incubated with the streptavidin-coated fluorescent beads for 3 hours at 37°C to directly coat the neoglycoconjugates on the fluorescent beads. The fluorescent beads adhesion assay was performed as described by Geijtenbeek *et al.*[25].

Soluble DC-SIGN-Fc adhesion assay

DC-SIGN-Fc consists of the extracellular portion of DC-SIGN (amino acid residues 64-404) fused at the C-terminus to a human IgG1-Fc fragment[22]. The soluble DC-SIGN adhesion assay was performed as follows. Soluble ligands were coated onto ELISA plates (1 μg/well) for 18 hours at room temperature, followed by blocking with 1% BSA for 2 hours at 37°C. Soluble DC-SIGN-Fc supernatant was added for 30 minutes at 37°C. Unbound DC-SIGN-Fc was washed away and binding was determined by anti-IgG1 ELISA. Specificity was determined in the presence of either 50 μg/ml blocking antibodies, 50 μg/ml mannan or 5 mM EGTA.

Immunofluorescence microscopy

Cells were incubated at 37°C with ManLAM (10 μg/ml). After 3 hours the cells were washed and incubated for an additional hour at 37°C. The cells were fixed in 3% paraformaldehyde in PBS for 15 minutes and permeabilized in PBS/0.1% saponin prior to staining. Cells were stained in PBS/0.5% BSA with antibodies against ManLAM (F30.5) and LAMP-1 and subsequently with Alexa Fluor 488 or 594-conjugated secondary antibodies. Next, cells were allowed to adhere to poly-L-lysine coated glass slides and mounted in anti-bleach reagent. Fixed slides were imaged with a Nikon Eclipse E800 fluorescence microscope and pictures were captured with a digital NIKON DXM1200 camera at 40× objective. Images were acquired in three independent series/sessions. Pictures were analyzed with Jasc Paint Shop Pro™ software.

3.3 Results

DC-SIGN interacts with the mannose cap of ManLAM

Recently, we have demonstrated that DC-SIGN is a major receptor on immature DC for mycobacteria, such as *M. tuberculosis* and *M. bovis* BCG[1]. The cell wall of my-

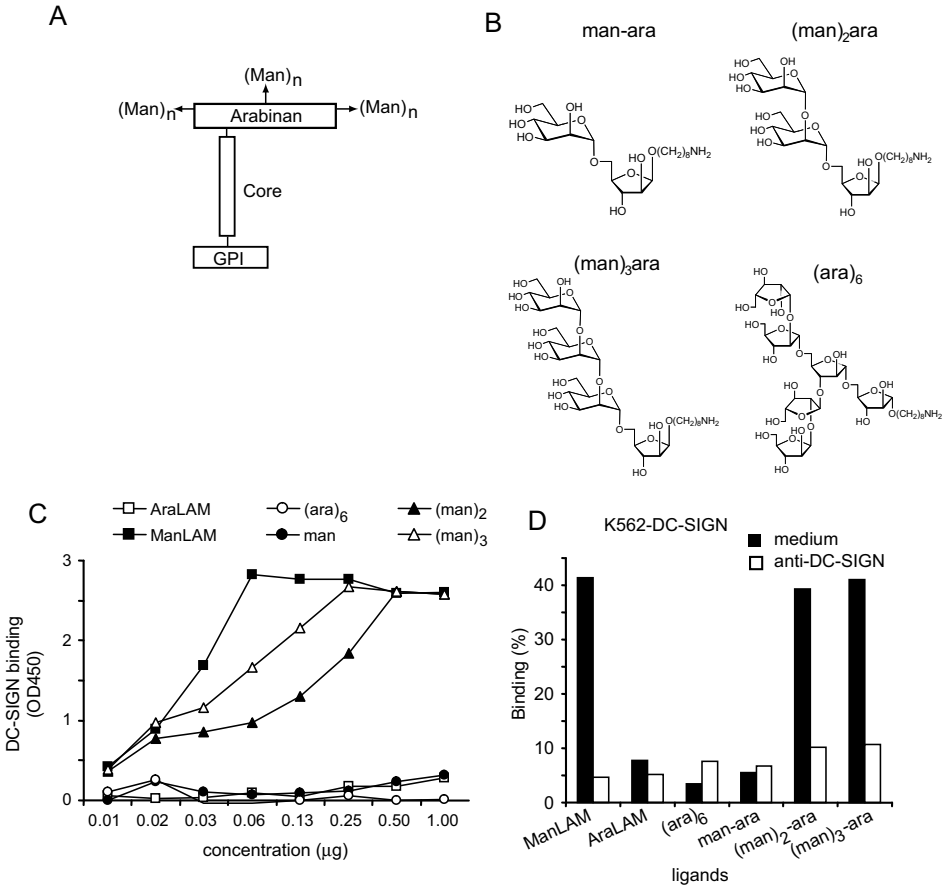

Figure 3.1: DC-SIGN interacts with the mannose cap of ManLAM

(A) The schematic structure of ManLAM. LAM of *M. tuberculosis* consists of a GPI anchor, a mannose-rich oligosaccharide core and a branched arabinose polymer that ends in mannose caps (n=0-3). AraLAM has a similar structure but does not contain the mannose cap. (B) Structures of the synthesized oligosaccharides present in the mannose cap of ManLAM. (C) DC-SIGN specifically interacts with $(man)_2$-ara and $(man)_3$-ara present in ManLAM. DC-SIGN-Fc binding to the PAA neoglycoconjugates was determined by an Fc-specific ELISA. Standard deviation < 0.02 OD450. One representative experiment out of three is shown. (D) Cellular DC-SIGN interacts specifically with the PAA neoglycoconjugates $(man)_2$-ara and $(man)_3$-ara. The neoglcyconjugates were coated on fluorescent beads and the adhesion to DC-SIGN transfectants was determined. The adhesion of cells to the LAM glycans was determined using the fluorescent bead adhesion assay. Specificity was determined by measuring binding in the presence of blocking antibodies against DC-SIGN. Standard deviation for the fluorescent bead adhesion assay was $< 5\%$. One representative experiment out of three is shown.

cobacteria contains LAM that comprises a mannose-rich polysaccharide-core, containing a highly branched arabinofuranosyl domain, and a glycosylphosphatidylinocitol (GPI) anchor (Fig. 3.1A)[8]. Both *M. tuberculosis* and *M. bovis* BCG contain ManLAM and the interaction of DC-SIGN with these mycobacterial strains was due to the binding of DC-SIGN to the mycobacterial cell wall[1]. In contrast, DC-SIGN does not bind to LAM lacking this mannose cap (AraLAM)[1], as demonstrated by the data that show that DC-SIGN does not interact with mycobacteria that contain AraLAM but not ManLAM, such as *M. smegmatis*[1,5]. ManLAM isolated from *M. tuberculosis* contains mannose-residues consisting exclusively of mono-, di- and trimers of α-D-mannopyranoses directly linked to the arabinofuranosyl-termini (Fig. 3.1B), whereas AraLAM isolated from the fast growing *M. smegmatis* is not mannose capped[8]. DC-SIGN has a high affinity for mannose-containing carbohydrates[12,19] and the differences in binding to ManLAM and AraLAM suggests that the mannose cap is recognized by DC-SIGN. We have synthesized specific LAM oligosaccharides consisting of mono-, di- and trimers of α-D-mannoses directly linked to the arabinofuranose moiety to investigate the interaction with DC-SIGN (Fig. 3.1C). These mannose structures were covalently linked to biotinylated PAA as a carrier. The structures are called $(\text{ara})_6$, man-ara, $(\text{man})_2$-ara and $(\text{man})_3$-ara and the PAA carrier contains 50 oligosaccharides per polymeric chain.

The interaction of DC-SIGN with the different synthesized neoglycoconjugates was investigated in detail using the DC-SIGN-Fc binding assay[22]. This assay is suitable to measure low avidity interactions. As demonstrated previously, DC-SIGN interacts efficiently with ManLAM but not with AraLAM (Fig. 3.1C)[1]. DC-SIGN interacts specifically with the $(\text{man})_2$-ara and $(\text{man})_3$-ara PAA neoglycoconjugates, whereas the C-type lectin does not bind the man-ara nor the control $(\text{ara})_6$ PAA neoglycoconjugates (Fig. 3.1C). DC-SIGN has the highest affinity for the $(\text{man})_3$-ara structures and even at high concentrations DC-SIGN does not interact with the monomer of α-D-mannose (Fig. 3.1C). Next, we investigated the interaction of cellular DC-SIGN with the various synthesized oligosaccharide structures. The biotin-PAA neoglycoconjugates were directly coated on fluorescent beads and the interaction with cells was investigated using the fluorescent bead adhesion assay. THP-1 transfectants stably expressing DC-SIGN interact with ManLAM but not with AraLAM (Fig. 3.1D). Analysis of the binding of DC-SIGN with the various synthesized structures demonstrates that DC-SIGN interacts specifically with the $(\text{man})_2$-ara and $(\text{man})_3$-ara structures; whereas DC-SIGN does not bind the man-ara and the control $(\text{ara})_6$ carbohydrates (Fig. 3.1D). The interaction with $(\text{man})_2$-ara and $(\text{man})_3$-ara was specific, since antibodies against DC-SIGN completely blocked the interaction of K562-DC-SIGN to the carbohydrates. These data demonstrate that both soluble and cellular DC-SIGN efficiently interact with the $(\text{man})_2$-ara and $(\text{man})_3$-ara as present in the mannose cap of ManLAM.

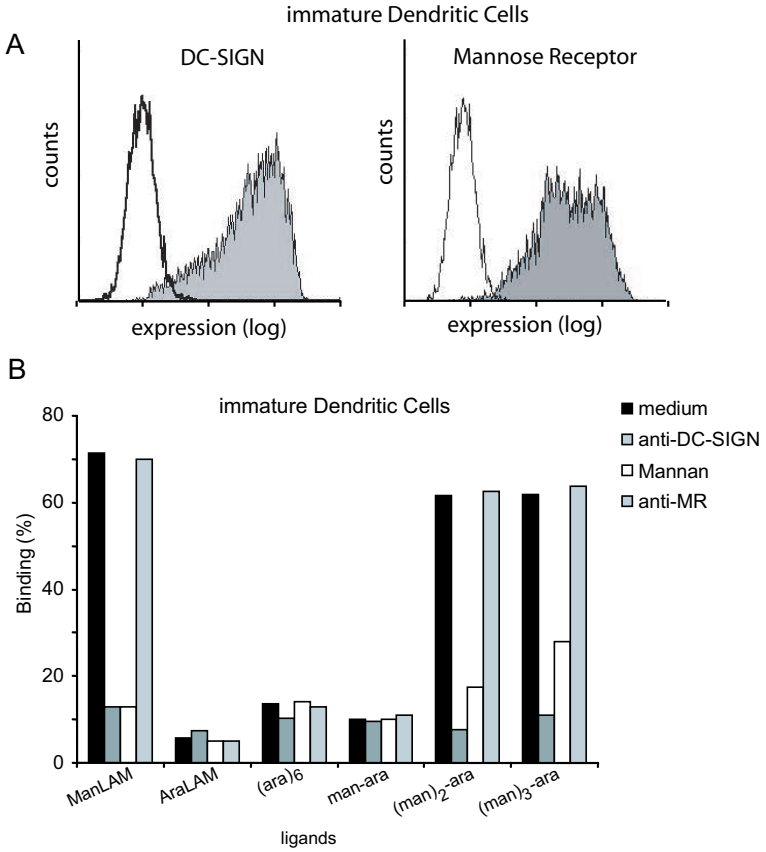

Figure 3.2: DC-SIGN is the major receptor for the mannose cap of ManLAM on DC
(A) Immature DC express high levels of DC-SIGN and the other reported LAM receptor MR. Open histograms represent isotype control and filled histograms indicate specific antibody staining. (B) Immature DC bind strongly to the PAA neoglycoconjugates $(\text{man})_2$-ara and $(\text{man})_3$-ara via DC-SIGN. Binding was determined using the fluorescent bead adhesion assay. Specificity was determined by measuring binding in the presence of mannan, or blocking antibodies against DC-SIGN (AZN-D2) and MR (Clone 19). Standard deviation $<5\%$. One representative experiment out of three is shown.

DC-SIGN is the major C-type lectin on DC for di- and trimers of α-D-mannoses as present in ManLAM

K562 cells do not express other receptors for mycobacteria such as MR that have been shown to interact with ManLAM[3,4]. However, immature DC express high levels of MR (Fig. 3.2A), which may interact with the synthetic carbohydrates. Therefore, we investigated the binding of DC with the various mannose-containing PAA neoglycoconjugates. Immature DC bind strongly to ManLAM and to the $(man)_2$-ara and $(man)_3$-ara structures, but not to the man-ara structures (Fig. 3.2B). The binding is completely blocked by antibodies against DC-SIGN, whereas the antibodies against MR do not block (Fig. 3.2B). The block with antibodies against DC-SIGN is similar to the inhibition with the polysaccharide mannan that blocks mannose-specific C-type lectins (Fig. 3.2B). These data demonstrate that DC-SIGN is the major receptor on immature DC for the PAA neoglycoconjugates $(man)_2$-ara and $(man)_3$-ara that are present in the mannose cap of ManLAM. Furthermore, these data demonstrate that the mannose cap present on ManLAM is indeed the structure that interacts with DC-SIGN, and that the C-type lectin recognizes the di- and trimers of α-D-mannoses directly linked to the arabinofuranosyl-termini, as present in *M. tuberculosis* ManLAM.

L-SIGN and mSIGNR1 interact with ManLAM

DC-SIGN has a homologue called L-SIGN[21,26] that is specifically expressed by liver sinusoidal endothelial cells (LSEC), a liver-resident antigen presenting cell and by endothelial cells in lymph nodes[21,27]. This homologue functions as an HIV-1 *trans*-receptor similar to DC-SIGN[21]. Moreover, L-SIGN interacts with other pathogens such as Ebola virus[28], cytomegalovirus[29] and hepatitis C virus[30,31] similar to DC-SIGN.

Next, we investigated the interaction of the DC-SIGN homologue L-SIGN and the murine homologue SIGNR1 with mycobacterial LAM. mSIGNR1 is a murine homologue of DC-SIGN that functions in vivo as a pathogen receptor for blood-borne antigens[20], and analysis of its pathogen specificity may help developing in vivo models for investigating the in vivo role of DC-SIGN in infections.

We have used THP-1 transfectants expressing similar levels of L-SIGN and mSIGNR1 to investigate the interaction with ManLAM and AraLAM (Fig. 3.3A). Both L-SIGN and mSIGNR1 bind strongly to ManLAM but not to AraLAM, as was observed for DC-SIGN (Fig. 3.3B). The interaction with ManLAM is specific for the C-type lectins, since the mock transfectant does not bind ManLAM. Moreover, the interaction could be completely inhibited by the polysaccharide mannan and EGTA, demonstrating that the interaction is mediated by the C-type lectin domain of both receptors.

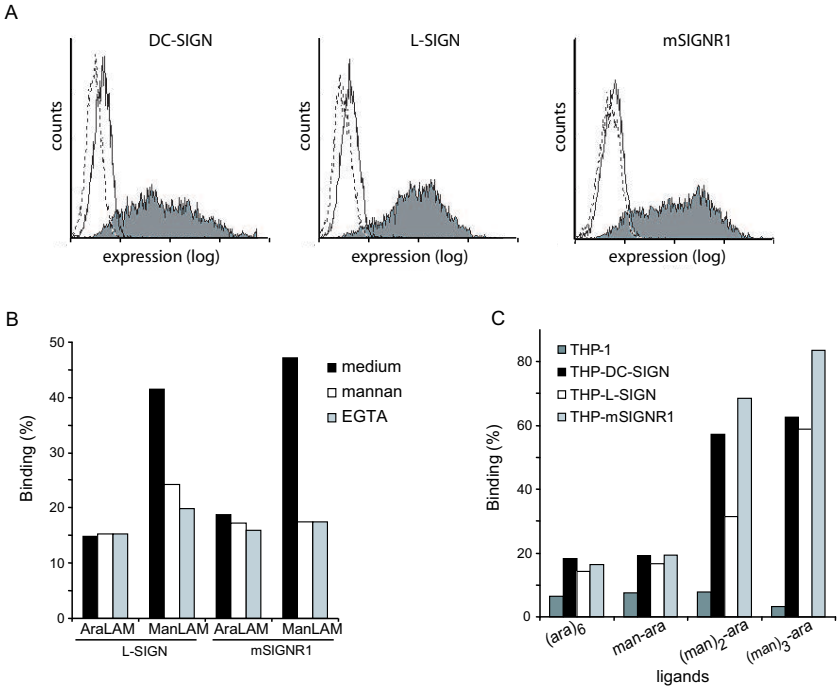

Figure 3.3: Both L-SIGN and mSIGNR1 bind strongly to the mannose cap of the mycobacterial component ManLAM

(A) THP-1 transfectants express similar levels of DC-SIGN, L-SIGN and mSIGNR1. Open histograms represent the isotype controls, dotted line represents mock transfectant, and filled histograms indicate the specific antibody staining. (B) Both L-SIGN and mSIGNR1 bind strongly to ManLAM but not to AraLAM. The adhesion of cells to the LAM glycans was determined using the fluorescent bead adhesion assay. Specificity was determined by the polysaccharide mannan and the chelator EGTA. Standard deviation for the fluorescent bead adhesion assay was $< 5\%$. One representative experiment out of three is shown. (C) The DC-SIGN homologues bind efficiently to the oligosaccharides $(\text{man})_2$-ara and $(\text{man})_3$-ara present on ManLAM. The adhesion of cells to the LAM glycans was determined using the fluorescent bead adhesion assay. Standard deviation for the fluorescent bead adhesion assay was $< 5\%$. One representative experiment out of three is shown.

L-SIGN and mSIGNR1 interact with oligosaccharides present in ManLAM

Analysis of the interaction of the DC-SIGN homologues with the synthetic LAM oligosaccharides in a fluorescent bead adhesion assay demonstrates that both L-SIGN and mSIGNR1 interact with $(\text{man})_2$-ara and $(\text{man})_3$-ara, similar to DC-SIGN (Fig. 3.3C). Both homologues do not interact with man-ara and the control oligosaccharide $(\text{ara})_6$. These data demonstrate that the C-type lectins L-SIGN and mSIGNR1 may act as receptors for ManLAM and that the interaction is mediated by both di- and tri-D-mannoses

present in ManLAM.

The amount of oligosaccharides coated on fluorescent beads is constant and in order to investigate the affinity of the C-type lectins for the different oligosaccharides, we used soluble oligosaccharide-coated PAA compounds labeled with biotin (data not shown). DC-SIGN, L-SIGN and mSIGNR1 have the highest affinity for the $(man)_3$-ara oligosaccharide (data not shown). These results demonstrate that the homologues of DC-SIGN, L-SIGN and mSIGNR1, behave similarly as DC-SIGN in their interaction with the mycobacterial oligosaccharides.

ManLAM is internalized by L-SIGN and mSIGNR1 and targeted to lysosomes

Recently, we have demonstrated that DC-SIGN internalizes antigens and targets them to lysosomal compartments for presentation on MHC Class II [32]. Similarly, fluorescent mycobacteria and ManLAM were captured by DC-SIGN on immature DC and targeted to the lysosomes [1]. Strikingly, the hepatitis C virus is targeted to the early endosomes by DC-SIGN expressed by DC and THP-1 transfectants [31]. The homologues L-SIGN and mSIGNR1 have different cytoplasmic domains containing different intracellular signaling motifs. Therefore, we investigated the fate of captured ManLAM by the DC-SIGN homologues expressed by THP-1 transfectants. ManLAM was efficiently captured and internalized by DC-SIGN, L-SIGN and mSIGNR1 on THP-1 transfectants, but not by mock transfectants (Fig. 3.4). Internalized ManLAM co-localized with the lysosomal marker CD107a/LAMP-1 in both THP-L-SIGN and THP-SIGNR1, similar to THP-DC-SIGN (Fig. 3.4) indicating that internalized ManLAM is targeted to $CD107a^+$ lysosomes by the DC-SIGN homologues.

3.4 Discussion

DC are vital in the defense against pathogens, but it is now becoming evident that some pathogens subvert DC functions to escape immunosurveillance. HIV-1 targets the DC-specific C-type lectin DC-SIGN to hijack DC for viral dissemination, whereas *M. tuberculosis* targets DC-SIGN by a mechanism that is distinct from that of HIV-1, leading to inhibition of the cellular function of DC, resulting in pathogen survival. Moreover, recent studies have demonstrated that DC-SIGN is a more universal pathogen receptor that also recognizes Ebola virus, cytomegalovirus and hepatitis C virus [11]. These studies indicate that DC-SIGN may be targeted by these pathogens to modulate immune responses in favor of their survival. Thus, a better understanding of the DC-SIGN-pathogen interactions is necessary to combat infections. However, DC-SIGN is involved in important cellular functions of the DC, such as initiating DC-T cell interactions [12] and DC migration [13]. Inhibiting DC-SIGN function by blocking antibodies against DC-SIGN may prevent the immunological function of DC-SIGN. Preventing the DC-SIGN-pathogen interaction through antibodies against pathogen-derived structures may provide an alternative

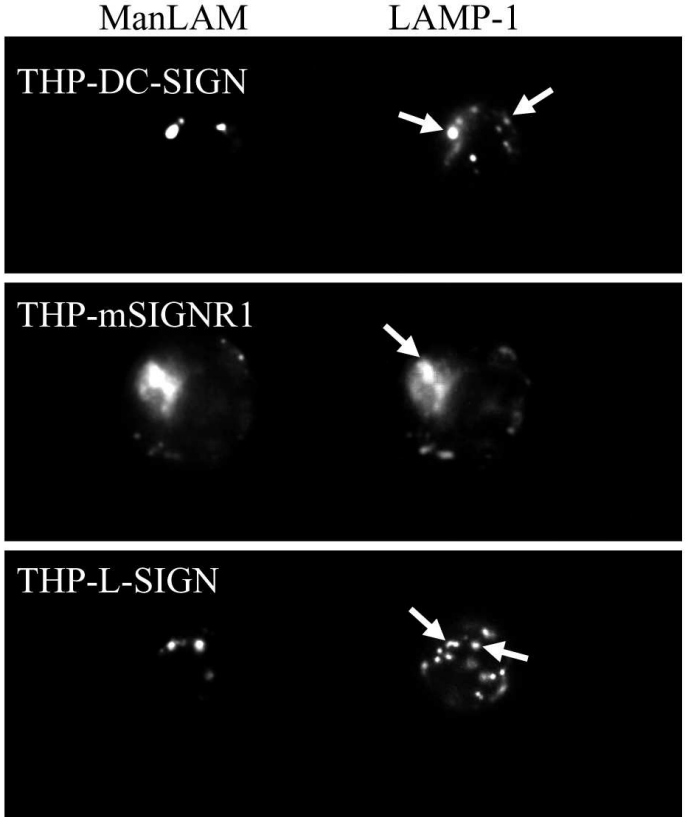

Figure 3.4: Both L-SIGN and mSIGNR1 mediate internalization of captured ManLAM
ManLAM is internalized by DC-SIGN, L-SIGN and mSIGNR1 and targeted to the lysosomes. The fate of captured ManLAM was followed by incubating transfectants with ManLAM (10 μg/ml) for 4 hours. ManLAM and CD107a/Lamp-1 were stained with F30.5 and H4A3, respectively. Arrows point towards co-localization of ManLAM with Lamp-1 vesicles. One representative experiment out of three is shown.

to DC-SIGN inhibitors. Therefore, the specific structures that are recognized by DC-SIGN need to be identified. DC-SIGN is a C-type lectin that has a high specificity for mannose-containing carbohydrates that are present in various pathogens [16,17,33]. As reported earlier, DC-SIGN binds to purified yeast mannan and high mannose-containing viral proteins [14,28,34] but also to less complex mannose-containing glycoconjugates i.e. mannose and $\alpha 1 \rightarrow 3, \alpha 1 \rightarrow 6$mannotriose [17,19]. Although these structures may be present on pathogens, so far the specific pathogenic carbohydrate structures that are recognized by DC-SIGN have not been identified.

Recently, we and others have demonstrated that *M. tuberculosis* binds to immature DC through DC-SIGN[1,5]. DC-SIGN binding to *M. tuberculosis* was mediated by ManLAM, since DC-SIGN did not interact with *M. smegmatis* that does not express ManLAM[1]. Moreover, DC-SIGN binds strongly to ManLAM- but not AraLAM-coated beads (Fig. 3.1). These data suggest that DC-SIGN interacts with the mannose cap of ManLAM. Indeed, removal of the mannose cap from ManLAM abrogates DC-SIGN binding [18]. ManLAM comprises a mannose-rich polysaccharide-core, containing highly branched arabinofuranosyl side chains, and a GPI anchor (Fig. 3.1)[8]. The mannose cap from *M. tuberculosis* contains mannose-residues consisting exclusively of mono-, di- and trimers of α-D-mannoses directly linked to the arabinofuranosyl-termini[8]. We have synthesized the mannose cap oligosaccharides man-ara, $(man)_2$-ara and $(man)_3$-ara, and covalently linked them to a PAA carrier. In these synthetic neoglycoconjugates, the mannose residues are bound to each other by α1→2 linkages, identical to the mannose cap of ManLAM. Both soluble and cellular DC-SIGN strongly interact with the $(man)_2$-ara and $(man)_3$-ara PAA glycoconjugates coated on fluorescent beads, whereas DC-SIGN does not bind to the man-ara glycoconjugates (Fig. 3.1). DC-SIGN has the highest affinity for the $(man)_3$-ara PAA neoglycoconjugate, as demonstrated by the binding assay using soluble PAA neoglycoconjugates (data not shown). Even though DC express various C-type lectins such as MR, the binding to the PAA neoglycoconjugates $(man)_2$-ara and $(man)_3$-ara is mediated by DC-SIGN, since the antibodies against DC-SIGN completely block the interaction (Fig. 3.2). These data support the role for DC-SIGN as a major receptor on immature DC for *M. tuberculosis* and demonstrate that $(man)_2$-ara and $(man)_3$-ara may represent targets to block the interaction of DC-SIGN with mycobacteria.

Recently, we have demonstrated that ManLAM blocks LPS-induced DC maturation and induces IL-10 production through binding of DC-SIGN[1]. In contrast, the PAA neoglycoconjugates did not block LPS-induced DC maturation or induce IL-10 production by binding to DC-SIGN (unpublished observations). This indicates that ManLAM contains more structures that are necessary for its signaling function. This is in agreement with the data from Nigou *et al.* that the GPI anchor on ManLAM is necessary for the inhibition of IL-12 production, presumably by binding of the fatty acyl chains to TLR4[(2)].

L-SIGN is a homologue of DC-SIGN that is not expressed by DC but by LSEC and endothelial cells in the lymph nodes[21,26]. The mRNA of L-SIGN shows about 90% similarity with DC-SIGN, and several studies have shown that L-SIGN has a similar binding specificity as DC-SIGN. L-SIGN binds to the cellular ligands of DC-SIGN i.e. ICAM-2 and ICAM-3[(21)] and to the envelope glycoproteins from HIV-1, Ebola virus, hepatitis C virus and cytomegalovirus[21,28–31]. L-SIGN has been demonstrated to function as a *trans*-receptor for HIV-1, similar to DC-SIGN, and therefore pathogens may target L-SIGN to escape immunosurveillance. Here, we have demonstrated that L-SIGN binds mycobacterial ManLAM but not AraLAM, suggesting that L-SIGN may bind *M. tuberculosis*. Binding assays with the PAA neoglycoconjugates demonstrate that L-SIGN interacts strongly with $(man)_2$-ara and $(man)_3$-ara, but not with man-ara, similar to DC-SIGN. These results

suggest that L-SIGN may be involved in the pathogenesis of *M. tuberculosis* infection, and demonstrate that L-SIGN captures ManLAM and rapidly internalizes it to the lysosomes. Thus, L-SIGN may be involved in the clearance of mycobacteria since L-SIGN is expressed on those sites in lymph nodes and liver that are ideally suited for antigen capture and clearance. However, mycobacteria may target L-SIGN to invade those tissues. More research is necessary to investigate the specific role of L-SIGN in these infections. Therefore, we have investigated the interaction of the murine DC-SIGN homologue with ManLAM.

mSIGNR1, a murine homologue of DC-SIGN, functions in vivo as a pathogen recognition receptor on marginal zone macrophages that captures blood-borne antigens, which are rapidly internalized and targeted to lysosomes for processing [20]. The C-type lectin domain of mSIGNR1 has a 74% similarity to DC-SIGN and also binds to HIV-1 gp120, and the cellular ligands of DC-SIGN, i.e. ICAM-2 and ICAM-3 [20]. Here, we demonstrate that mSIGNR1 binds mycobacterial ManLAM through the mannose cap (Fig. 3.3). Furthermore, mSIGNR1 interacts specifically with the PAA neoglycoconjugates $(man)_2$-ara and $(man)_3$-ara, but not with man-ara. The similar binding specificity to DC-SIGN suggests that the binding of mSIGNR1 to *M. bovis* BCG is mediated by the cell wall component ManLAM. Moreover, the similar binding characteristics of mSIGNR1 and DC-SIGN will enable us to design and test in mouse models in vivo vaccines that will prevent the interaction of DC-SIGN with pathogens such as mycobacteria. The function of mSIGNR1 is inhibited in vivo by the antibody ERTR-9 which will enable us to investigate the in vivo function of mSIGNR1 in *M. tuberculosis* infections.

The identification of the specific carbohydrate structures on *M. tuberculosis* that interact with DC-SIGN will enable us to develop strategies to specifically interfere with this interaction without blocking the immunological function of DC-SIGN.

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Chapter 4

Mice lacking murine SIGNR1 have a normal host defense against *Mycobacterium tuberculosis*

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Submitted

Abstract

Mycobacterium tuberculosis and the associated disease tuberculosis is a health risk causing many deaths worldwide each year in humans. *M. tuberculosis* targets dendritic cell-specific intercellular adhesion molecule-3 grabbing nonintegrin (DC-SIGN) to induce immunosuppression, since interaction of DC-SIGN with mycobacterial mannose-capped lipoarabinomannan (ManLAM) induces interleukin (IL)-10 and prevents DC maturation. We investigated the role of a murine homologue of DC-SIGN, SIGN Related 1 (mSIGNR1), in a model of *M. tuberculosis* infection using mSIGNR1-deficient (KO) mice. In contrast to DC-specific DC-SIGN, mSIGNR1 is expressed by macrophage subpopulations in lymph node, spleen and peritoneal cavity and not by alveolar macrophages even during infection. As a consequence, we did not detect any differences between wild-type (WT) and KO mice in mycobacterial loads of lung, spleen, liver and draining lymph node after 1 day, 2 and 5 weeks of *M. tuberculosis* infection resulting in similar survival rates. Although pulmonary levels of IL-10 and interferon (IFN)γ were similar in both groups, we observed increased T cell activity in mSIGNR1 KO mice early during infection and increased IFNγ production by splenocytes in mSIGNR1 KO mice early in infection suggesting an immunomodulatory role for mSIGNR1. This is in agreement with in vitro data demonstrating that peritoneal macrophages from mSIGNR1 KO mice produce significantly less IL-10 upon stimulation with ManLAM than those from WT mice, suggesting that the interaction of ManLAM with mSIGNR1 can result in immunosuppression similar to its human homologue. Nevertheless, our data suggest that the role of mSIGNR1 is limited during murine *M. tuberculosis* infection.

4.1 Introduction

Mycobacterium tuberculosis and the associated disease tuberculosis are main threats to mankind, with one-third of the world population being infected[1]. Over 8 million new cases of tuberculosis and 2 million deaths from this disease occur yearly worldwide[2]. Although healthy persons infected with *M. tuberculosis* develop an immune response, this response is not strong enough to eradicate the bacterium. Mechanisms by which *M. tuberculosis* escapes from the host immune response have been studied in detail. Although macrophages, and not dendritic cells (DC), are the primary targets for infection by mycobacteria, DC are important for the cellular immune response. Recent data strongly suggest that C-type lectins are involved in suppressing cellular immune responses mediated by DC[3,4].

C-type lectins recognize pathogens by binding to pathogen-specific carbohydrate residues. The human C-type lectin DC-SIGN, which is expressed by DC[5], binds to ManLAM, a major cell wall component of *M. tuberculosis*[6–8]. Both membrane bound and secreted ManLAM are considered an important virulence factor of *M. tuberculosis*[9,10].

After binding to DC-SIGN, ManLAM stimulates the production of the anti-inflammatory cytokine interleukin (IL)-10 by DC and inhibits Toll-like receptor-induced DC maturation [6]. These findings indicate that in humans, binding of ManLAM to DC-SIGN hampers DC function leading to suppression of the adaptive immune response against *M. tuberculosis* and thereby possibly facilitates survival of the pathogen.

It was recently shown that 70% of alveolar macrophages obtained from patients with tuberculosis expressed DC-SIGN on their surface and this expression was not present in alveolar macrophages from patients with non-mycobacterial lung pathologies or from healthy controls [11]. Hence, DC-SIGN, expressed by DC and specific macrophage subsets, could play a role in the immune defense against *M. tuberculosis*.

Five different homologue genes of DC SIGN were cloned in mice but the cellular expression has been elucidated only for two of these murine homologues: murine DC-SIGN (mDC-SIGN) and murine SIGN Related 1 (mSIGNR1) [12–15]. mDC-SIGN is expressed by plasmacytoid pre-DC [16,17]. However, no ligands for mDC-SIGN have been identified including known ligands for human DC-SIGN, suggesting that the murine homologue is inactive [16,18,19]. In contrast, mSIGNR1 is abundantly expressed in lymph node by medullary and subcapsular macrophages, in spleen by marginal zone macrophages and in the liver by sinusoidal endothelial cells [15]. In addition, Taylor *et al.* demonstrated that mSIGNR1 is also expressed on resident peritoneal macrophages [14]. The function and binding capacities of mSIGNR1 have been studied in detail and are similar to human DC-SIGN [7,15,20]. Recently, it has been demonstrated that mSIGNR1 is important in the defense against *Streptococcus pneumoniae* infection [21,22]. Interestingly, like DC-SIGN, mSIGNR1 binds to ManLAM and *M. tuberculosis* [7,14].

Here we set out to elucidate the in vivo function of mSIGNR1 in the immune defense against *M. tuberculosis*. The role of mSIGNR1 in the host defense response against *M. tuberculosis* was studied using mSIGNR1-deficient (KO) mice [21]. Upon stimulation with ManLAM, the production of IL-10 by wild-type (WT) peritoneal macrophages was induced. Strikingly, IL-10 production by KO peritoneal macrophages was reduced compared to WT, suggesting an immunosuppressive role for mSIGNR1 upon infection with *M. tuberculosis* similar to DC-SIGN. In addition, during the early stage of infection, splenocytes from KO mice produced more interferon (IFN)γ than WT splenic T cells, supporting the attenuated IL-10 response by peritoneal macrophages of KO mice in response to ManLAM. These data suggest a more pronounced Th1-specific immune response in the KO mice upon *M. tuberculosis* infection. However, no differences in susceptibility to *M. tuberculosis* were observed between mSIGNR1 KO and WT mice. Our data suggest that although mSIGNR1 is able to bind ManLAM and *M. tuberculosis* in vitro, its role is limited during *M. tuberculosis* infection in vivo.

4.2 Materials and Methods

Mice

C57BL/6×129 WT and mSIGNR1 KO mice were bred in the animal facility of the VU University Medical Center under specific pathogen-free conditions, and were kept in the animal facilities of the VU University Medical Center and the Academic Medical Center in Amsterdam, The Netherlands. Six- to eight-week-old male mice were used in the infection experiments. The Animal Care and Use Committee of the University of Amsterdam approved all experiments.

Preparation of peritoneal macrophages

Peritoneal macrophages were harvested from mSIGNR1 KO and WT mice (n= 4 to 6 per strain). Mice were sacrificed and peritoneal lavage was performed with 5 ml sterile isotonic saline supplemented with 50 IE/ml heparin, and peritoneal lavage fluid was collected in sterile tubes using a glass Pasteurs pipet. Total cell numbers were counted from each sample using a counting chamber. Cells were resuspended in RPMI 1640 containing 1 mM pyruvate, 2 mM L-glutamine, penicillin, streptomycin and 10% FCS in a final concentration of 2×10^4 cells/200 μl. Cells were cultured in 96-well microtiter plates (Greiner, Alphen a/d Rijn, The Netherlands) for 2 hours and washed with RPMI 1640 to remove non-adherent cells. Adherent cells were stimulated with ManLAM (10 μg/ml; obtained from Dr. J.T. Belisle from the Colorado State University, Fort Collins, CO (under NIH Contract NO1-AI-75320)) or with medium for 24 hours in a total volume of 100 μl. Supernatants were collected and stored at -20°C until assayed for IL-10.

Experimental infection

A virulent laboratory strain of *M. tuberculosis* H37Rv (American Type Culture Collection, Rockville, MA) was grown for 4 days in liquid Dubois medium containing 0.01% Tween-80. A replicate culture was incubated at 37°C, harvested at mid-log phase, and stored in aliquots at 70°C. For each experiment, a vial was thawed and washed with sterile 0.9% NaCl. Tuberculosis was induced as described previously [23,24]. Briefly, mice were anesthetized by inhalation with isoflurane (Abbott Laboratories Ltd., Kent, United Kingdom) and infected intranasally with 10^5 live *M. tuberculosis* H37Rv bacilli in 50 μl saline, as determined by viable counts on Middlebrook 7H11 plates. Groups of eight mice per time point were sacrificed two and five weeks after infection. Lungs, liver and spleen were removed aseptically and homogenized in 5 volumes of sterile 0.9% NaCl. 10-fold dilutions were plated on Middlebrook 7H11 agar plates to determine bacterial loads. Colonies were counted after 21 days at 37°C. Numbers of colony-forming units (CFU) are provided per gram of lungs. In order to check for infection efficacy, three mice per group were sacrificed one day post-infection. For cytokine measurements, organ ho-

mogenates were diluted 1:2 in lysis buffer containing 300 mM NaCl, 30 mM Tris, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 1% Triton X-100, and pepstatin A, leupeptin and aprotinin (all 20 ng/ml; pH 7.4) and incubated on ice for 30 minutes. Homogenates were centrifuged at $1500\times g$ at 4°C for 15 minutes, and supernatants were sterilized using a 0.22 μm filter (Corning Incorporated, Corning, NY) stored at -20°C until assays were performed.

Characterization of inflammatory infiltrates in the lungs

Lung and draining mediastinal lymph node cell suspensions were obtained by crushing through a 40-μm cell strainer (Becton Dickinson, Franklin Lakes, NJ) as described previously[23,24]. Erythrocytes in the cell suspensions were lysed with ice-cold isotonic NH_4Cl solution (155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA, pH 7.4), the remaining cells were washed twice with RPMI 1640 (Bio Whittaker, Verviers, Belgium), and counted by using a hemocytometer. The percentages of macrophages, polymorphonuclear cells (PMNs) and lymphocytes were determined using cytospin preparations stained with haematoxilin and eosin.

Flow cytometric analysis

Lung cell suspensions obtained from infected mice were analyzed by flow cytometry using a FACS Calibur (Becton Dickinson Immunocytometry Systems, San Jose, CA) as described previously[23,24]. Cells were brought to a concentration of 1×10^7 cells per ml of FACS buffer (PBS supplemented with 0.5% BSA, 0.01% NaN_3 and 0.35 mM EDTA). Immunostaining for cell surface molecules was performed for 30 minutes at 4°C using directly labeled antibodies against CD3 (CD3-phycoerythrin), CD4 (CD4-APC), CD8 ($CD8^-$ peridinin chlorophyl protein) and CD69 (CD69-FITC). All antibodies were used in concentrations recommended by the manufacturer (Pharmingen, San Diego, CA). After staining, cells were fixed in 2% paraformaldehyde, and T cell surface molecules were analyzed within the gate containing $CD3^+$ cells.

Histology

Lungs were removed 2 and 5 weeks after inoculation with *M. tuberculosis*, fixed in 10% buffered formaline for 24 hours, and embedded in paraffin. Hematoxilin and eosin stained slides were coded and scored from 0 (absent) to 4 (severe) for the following parameters: interstitial inflammation, endothelialitis, bronchitis, oedema, granuloma formation and pleuritis by a pathologist. In addition, the percentage of inflamed lung parenchyma was scored separately. For mSIGNR1 stainings the slides were deparaffinized; endogenous peroxidase activity was quenched by a solution of methanol/0.03% H_2O_2 (Merck, Darmstadt, Germany). After digestion with a 10 mM sodium citrate solution, pH 6.0, nonspecific binding was blocked with TENG-T (10 mM Tris, 5 mM EDTA, 0.15 M NaCl,

0.25% gelatine, 0.05% (v/v) Tween 20, pH 8) and then exposed to a goat anti-SIGNR1 antibody (R&D Systems, Minneapolis, MN). After washes, slides stained for mSIGNR1 were incubated with a horseradish peroxidase-labeled rabbit anti-goat IgG2a antibody (Southern Biotech, Birmingham, AL). Slides were finally developed using 1% H_2O_2 and 3,3'-diaminobenzidine-tetra-hydrochloride (Sigma) in Tris-HCl and slides were counter-stained with methyl green. The sections were mounted in glycerin gelatin. As a negative control, spleens and lungs from mSIGNR1 KO mice were stained.

Splenocyte stimulation

Single cell suspensions were obtained by crushing spleens through a 40-μm cell strainer (Becton Dickinson, Franklin Lakes, NJ) as described[23,25]. Erythrocytes were lysed with ice-cold isotonic NH_4Cl solution (155 mM NH_4Cl, 10 mM $KHCO_3$, 100 mM EDTA, pH 7.4); the remaining cells were washed twice with RPMI 1640 (Bio Whittaker, Verviers, Belgium) supplemented with 10% FCS and 1% antibiotic-antimycotic (GiboBRL, Life Technologies, Rockville, MD). Cells were seeded in 96-well round bottom culture plates at a cell density of 1×10^6 cells per well in quadruplicate, and stimulated with 20 μg/ml tuberculin purified protein derivative (PPD, Statens Seruminstitut, Copenhagen, Denmark). Supernatants were harvested after incubation for 48 hours at 37°C in 5% CO_2, and cytokine levels were analyzed by ELISA.

Cytokine measurements

The IL-10 in the stimulation assay was analyzed by Cytometric Bead Array (CBA, Pharmingen, San Diego, CA) according to the manufacturers instructions. The detection limit for IL-10 was 12.5 pg/ml. For measuring IFNγ, IL-4, tumor necrosis factor (TNF), IL-1β, IL-10, KC and MIP-2 in lung homogenates, ELISA using matched antibody pairs according to the manufacturers instructions was used (R&D Systems Inc., Minneapolis, Minnesota, USA). Detection limits were 63 pg/ml for IFNγ, IL-4, IL-10, TNF, IL-1β and MIP-2 and 15 pg/ml for KC.

Statistical analysis

All values are expressed as mean $\pm$ SEM. Comparisons were done with Mann-Whitney U tests using GraphPad Prism version 4.00, GraphPad Software (San Diego, CA). When comparing two groups at multiple time points two way ANOVA was used. Statistical analyses of bacterial counts were performed after log transformation. Values of $P<0.05$ were considered statistically significant.

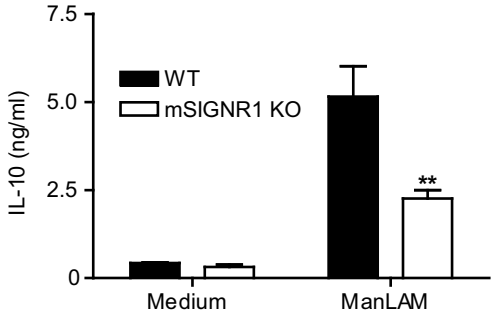

Figure 4.1: Reduced IL-10 production of mSIGNR1 KO peritoneal macrophages
Macrophages from WT (black bars) and mSIGNR1 KO (white bars) mice were harvested by lavage of the peritoneal cavity. After adherence, macrophages were stimulated with medium or ManLAM (10 μg/ml). After 24 hours of stimulation, IL-10 was determined in the supernatant. Data are presented as means $\pm$ SEM of 4-6 mice per group. $^{**}P<0.01$ versus WT.

4.3 Results

Reduced IL-10 production by mSIGNR1 KO peritoneal macrophages in response to ManLAM

Previously it has been shown that ManLAM, a component of the capsule of *M. tuberculosis*, is able to bind DC-SIGN expressed by human DC and this interaction induces an increase in IL-10 production after Toll-like receptor triggering[6]. Therefore, ManLAM, which is also secreted by *M. tuberculosis*, can act as an immunosuppressive agent facilitating infection of *M. tuberculosis*. In this study we set out to investigate the role of mSIGNR1, a murine homologue of DC-SIGN in the immune response against *M. tuberculosis* in an infection model. Murine resident peritoneal macrophages express mSIGNR1, which binds ManLAM[14]. In order to investigate whether ManLAM binding to mSIGNR1 results in a similar immunosuppressive cytokine response as has been observed with DC-SIGN, resident peritoneal macrophages from both WT and mSIGNR1 KO mice were stimulated with ManLAM. Indeed, mice lacking mSIGNR1 were less able to produce IL-10 in response to ManLAM (Fig. 4.1). Hence, ManLAM can exert its immunosuppressive effects through mSIGNR1 on peritoneal macrophages.

No differences in *M. tuberculosis* growth in lung, spleen or liver

To determine the role of mSIGNR1 in antibacterial defense against tuberculosis, the outgrowth of *M. tuberculosis* in lungs of mSIGNR1 KO and WT mice was determined after 1 day, 2 and 5 weeks after intranasal inoculation (Fig. 4.2). At all time points after infection, the numbers of CFU detected in the lung were similar in mSIGNR1 KO and WT

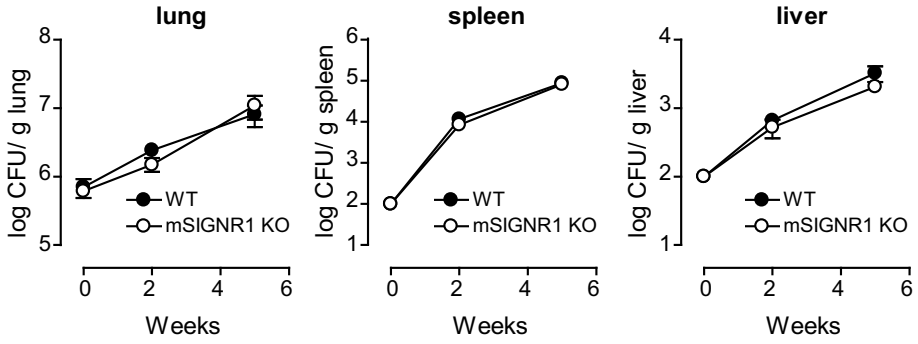

Figure 4.2: Unaltered bacterial outgrowth in mSIGNR1 KO mice
WT (closed symbols) and mSIGNR1 KO mice (open symbols) were infected intranasally with 10^5 CFU of *M. tuberculosis*. One day, 2 and 5 weeks after infection, mice were sacrificed and bacterial loads were determined in homogenates of lung, spleen and liver. Data are presented as means $\pm$ SEM of 3 (day 1) to 6-8 mice per group per time point.

animals. To study the dissemination of *M. tuberculosis*, the bacterial loads in spleen and liver were measured. No differences in bacterial growth in spleen or liver were observed between mSIGNR1 KO and WT mice at the different time points (Fig. 4.2). To further assess the role of mSIGNR1 in host defense against tuberculosis, 14 mSIGNR1 KO and 14 WT mice were studied during a 6 month observation period after infection. A similar percentage of animals died (8 out of 14 in each group; data not shown). Thus, mSIGNR1 does not influence the outgrowth or dissemination of *M. tuberculosis* nor does it appear to be essential for survival after infection with this mycobacterium.

No differences in histopathology between lungs of mSIGNR1 KO and WT mice

Two weeks after infection, lungs of both WT and mSIGNR1 KO mice already displayed advanced tuberculosis with high scores for interstitial inflammation and inflammation of vessels and bronchi (14.4$\pm$0.6 for WT and 14.6$\pm$1.9 for mSIGNR1 KO lungs; Fig. 4.3). The diffuse infiltrate consisted mainly of mononuclear cells and no differences in the degree of inflammation could be detected between WT and mSIGNR1 KO mice (Fig. 4.3 and Table 4.1). After 5 weeks of infection, lung inflammation scores increased (18.8$\pm$0.6 for WT and 17.2$\pm$1.7 for mSIGNR1 KO lungs). Moreover, the percentage of lung parenchyma involved in inflammation was higher at 5 weeks post-infection and this resulted in higher total cell counts in the lungs of both groups (Table 4.1). In contrast to 2 weeks post-infection, the percentage of infiltrated PMNs increased in the lungs of both groups after 5 weeks of infection (Table 4.1). Based on these results it seems that mSIGNR1 is not crucial for the inflammation of the lungs and the concomitant cellular infiltration, upon infection with *M. tuberculosis*.

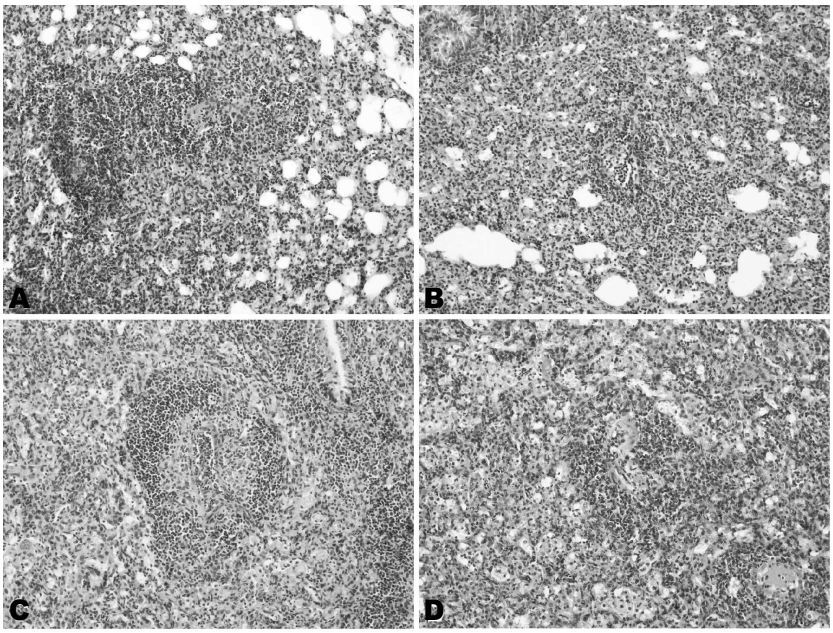

Figure 4.3: In both WT and mSIGNR1 KO mice a similar degree of inflammation was observed in the lung
(color reprint: Fig. A.1, pp. 154)
Representative lung histology of WT (A and C), and mSIGNR1 KO (B and D) mice, 2 (A, B) and 5 (C, D) weeks after intranasal infection with 10^5 CFU of *M. tuberculosis*. The lung sections are representative for 6-8 mice per group per time point. H&E staining, magnification $\times 10$.

	Cells $\times 10^5$/ml	Mϕs %	PMNs %	Lymphocytes %
2 weeks				
WT	40±6	42±3	25±4	32±4
mSIGNR1 KO	53±11	44±5	28±3	28±4
5 weeks				
WT	138±57	37±4	38±5	25±4
mSIGNR1 KO	122±43	38±7	34±7	15±3

Table 4.1: Effect of mSIGNR1 deficiency on total and differential lung cell counts
Total leukocyte counts ($\times 10^5$/ml) and differential cell counts in lungs of WT and mSIGNR1 KO mice 2 and 5 weeks after intranasal infection with 10^5 CFU of *M. tuberculosis*.

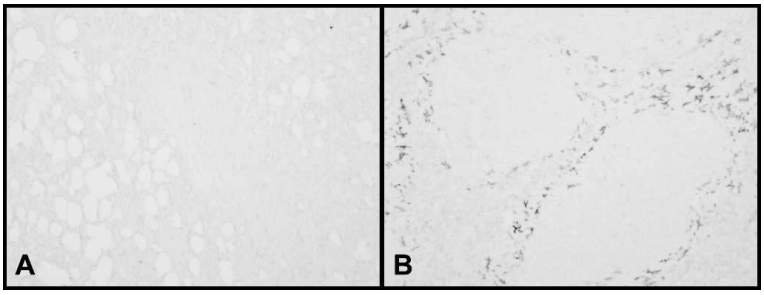

Figure 4.4: No expression of mSIGNR1 in lungs of infected WT mice
(color reprint: Fig. A.2, pp. 155)
Representative mSIGNR1 stainings of lung (A) and spleen (B) from WT mice 2 weeks after intranasal infection with 10^5 CFU of *M. tuberculosis*. No mSIGNR1 positive cells were detectable in the lung whereas marginal zone macrophages are stained positively. Sections are representative for 6-8 mice per group. Original magnification $\times 10$.

No induction of mSIGNR1 in the lung during *M. tuberculosis* infection

DC-SIGN is expressed by alveolar macrophages upon infection with *M. tuberculosis* [11]. Therefore we investigated whether mSIGNR1 is upregulated on alveolar macrophages during *M. tuberculosis* infection. Lung slides from WT and mSIGNR1 KO were stained with a polyclonal antibody against mSIGNR1. Lungs from infected WT mice did not demonstrate any positive staining at both 2 and 5 weeks after infection (Fig. 4.4A for 2 weeks and data not shown for 5 weeks). As expected, macrophages in the marginal zones of spleens from WT mice stained positive (Fig. 4.4B) and no positive staining for mSIGNR1 was visible in spleens from mSIGNR1 KO mice demonstrating the specificity of the antibody used (data not shown). Thus, inflammation due to infection by *M. tuberculosis* does not induce the expression of mSIGNR1 in macrophages of the lung and demonstrates further that mSIGNR1 and DC-SIGN have a different expression pattern.

mSIGNR1 KO splenocytes produce increased IFNγ levels early in infection with unchanged T cell recruitment

The Th1 response is pivotal for an adequate immune response against *M. tuberculosis*. Therefore, we compared concentrations of IFNγ and IL-4 as prototypic Th1 and Th2 cytokine in lung homogenates and after splenocyte stimulation with the *M. tuberculosis* specific antigen PPD (Fig. 4.5). Moreover we studied T cell subsets in lungs and local lymph node at 2 and 5 weeks after infection (Table 4.2). Absence of mSIGNR1 resulted in increased production of the Th1 cytokine IFNγ and reduced the production of IL-4 by splenocytes after 48 hours of stimulation with PPD (Fig. 4.5). No differences in local IFNγ and IL-4 concentrations were detected (Table 4.3). T cell subset recruitment was

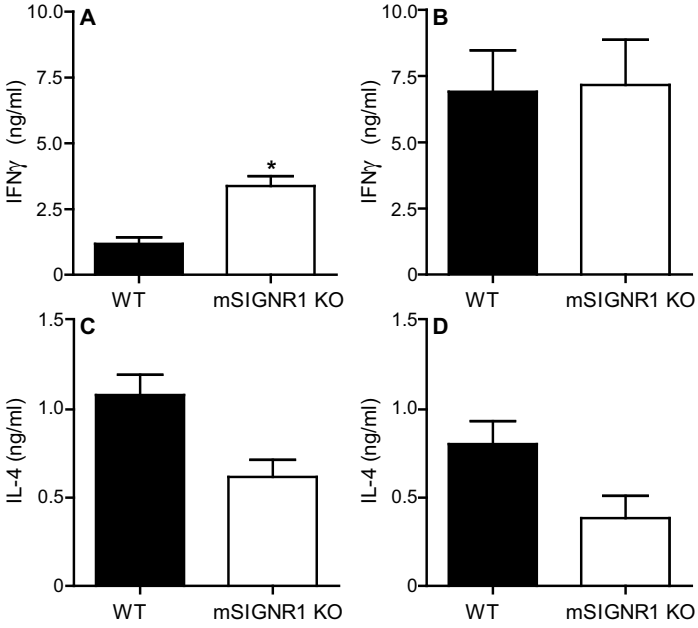

Figure 4.5: Enhanced early antigen specific IFNγ response by splenocytes from infected mSIGNR1 KO mice

Splenocytes were isolated from WT (filled bars) and mSIGNR1 KO mice (open bars) 2 (A, C) and 5 (B, D) weeks after infection with 10^5 *M. tuberculosis* and stimulated with PPD for 48 hours. IFNγ (A, B) and IL-4 (C; D) were measured in culture supernatants. Data are mean ± SEM of 6-8 mice per group per time point. $^*P < 0.05$ versus WT mice.

equal in lungs and draining lymph node of WT and mSIGNR1 KO mice (Table 4.2). Nevertheless, the early activation status (% CD69 positive lymphocytes) of lung $CD8^+$ T cells and draining lymph node $CD4^+$ T cells was increased after 2 weeks but not after 5 weeks of infection in the mSIGNR1 KO mice compared to the WT mice. The increase in IFNγ and T cell activation status in the mSIGNR1 KO mice compared to WT mice indicates that mSIGNR1 might be involved in the shift of the balance of the early immune response against *M. tuberculosis* towards Th2.

Similar local cytokine and chemokine levels

It has been demonstrated that in vitro binding of *M. tuberculosis* ManLAM to DC-SIGN expressed on DC and mSIGNR1 expressed on macrophages results in production of the anti-inflammatory cytokine IL-$10^{(6)}$ (Fig. 4.1). We therefore assessed the local inflammatory response in WT and mSIGNR1 KO mice infected with *M. tuberculosis*. De-

Lung	$CD4^+$ %	$CD4^+/CD69^+$ %	$CD4^+$ %	$CD8^+/CD69^+$ %
2 weeks				
WT	79±3	29±2	13±2	5±1
mSIGNR1 KO	73±2	26±3	17±2	9±1*
5 weeks				
WT	82±3	29±3	11±3	5±2
mSIGNR1 KO	88±1	34±3	8±1	4±1
Lymph node				
2 weeks				
WT	62±2	10±1	35±2	5±1
mSIGNR1 KO	63±2	17±3*	31±2	5±1
5 weeks				
WT	87±3	13±3	10±3	1±1
mSIGNR1 KO	83±6	14±1	10±2	2±1

Table 4.2: Effect of mSIGNR1 deficiency on T cell subsets in lungs during tuberculosis
T lymphocyte subsets in lungs or draining mediastinal lymph nodes of WT and mSIGNR1 KO mice, 2 and 5 weeks after intranasal infection with 10^5 CFU of *M. tuberculosis*. Data are presented as the percentage positive cells in the $CD3^+$ gate. Data are mean ± SEM of 6-8 mice per group. *$P<0.05$ versus WT mice.

spite the fact that ManLAM induced IL-10 production by DC and peritoneal macrophages in vitro, no differences in IL-10 production were observed in the lung during pulmonary tuberculosis in mice deficient for mSIGNR1 compared to WT mice. As shown in Table 4.3, no differences in total pulmonary cytokine and chemokine levels were observed: in addition to IL-10, TNF, IL-1β, KC and MIP-2 concentrations were comparable in WT and mSIGNR1 KO mice. Based on the cytokine profile measured locally, mSIGNR1 is not involved in the induction of cytokine production to modulate the immune response.

4.4 Discussion

Early interactions between DC and *M. tuberculosis* are thought to be critical for mounting a protective anti-mycobacterial immune response. Recent studies have demonstrated that *M. tuberculosis* is able to bind to DC-SIGN, a C-type lectin expressed on DC[6,8]. The interaction of the *M. tuberculosis* cell wall component ManLAM with DC-SIGN resulted in suppression of LPS-induced DC maturation and production of the immunosuppressive cytokine IL-10 in vitro[6]. These data suggest that *M. tuberculosis* targets the C-type lectin DC-SIGN to suppress DC functions[6]. In vivo studies are needed to fully understand the role of DC-SIGN in *M. tuberculosis* infection.

	2 weeks		5 weeks	
ng/ml	WT	mSIGNR1 KO	WT	mSIGNR1 KO
IFNγ	0.4±0.1	0.5±0.1	0.4±0.1	0.2±0.1
IL-4	0.4±0.1	0.5±0.1	0.5±0.1	0.8±0.1
IL-10	2.2±0.2	3.3±0.4	4.2±0.2	3.1±1.0
TNF	3.2±0.7	3.9±0.3	4.1±0.3	5.9±1.1
IL-1β	2.2±0.5	1.7±0.2	1.6±0.3	1.7±0.3
KC	4.7±0.9	4.0±0.7	4.3±0.8	3.4±1.0
MIP-2	0.3±0.1	0.3±0.1	0.6±0.1	0.4±0.1

Table 4.3: No effect of mSIGNR1 deficiency on pulmonary cytokine and chemokine levels
Cytokine and chemokine levels (in ng/ml) in lung homogenates of WT and mSIGNR1 KO mice 2 and 5 weeks after intranasal infection with 10^5 CFU of *M. tuberculosis*. Data are mean ± SEM of 6-8 mice per group.

Here, we have studied the role of mSIGNR1 in a model of pulmonary tuberculosis. Although this homologue is capable of binding both *M. tuberculosis* and ManLAM, similar to human DC-SIGN, it is expressed by macrophages and not DC. We found that mSIGNR1 expressed by peritoneal macrophages is involved in ManLAM-induced IL-10 production. Therefore, we expected that the absence of the ManLAM induced down-modulation of the immune response by the production of IL-10 would lead to an enhanced host defense response against *M. tuberculosis* in mSIGNR1 KO mice. In contrast to our hypothesis, we were not able to detect any differences in local pulmonary concentrations of IL-10, IFNγ or other cytokines at 2 and 5 weeks post-infection. Moreover, no differences were observed between mSIGNR1 KO and WT mice with respect to the numbers of pulmonary $CD4^+$ and $CD8^+$ T lymphocytes at 2 and 5 weeks after infection. We did observe increased activation in pulmonary $CD8^+$ T cells and lymph node $CD4^+$ cells early after infection in mSIGNR1 KO mice compared to the WT. However, the increased expression of CD69 in these cells did not lead to increased local levels of IFNγ, the protective cytokine necessary in host defense against *M. tuberculosis* [26,27].

Strikingly, at 2 weeks after infection, splenocytes from mSIGNR1 KO mice produced more IFNγ upon stimulation with the recall antigen PPD compared to WT mice. This indicates that the systemic immune response was enhanced at this early time-point in mSIGNR1 KO mice compared to the WT suggesting an immunosuppressive role for mSIGNR1. This immunosuppressive role of mSIGNR1 is confirmed by the trend that stimulated mSIGNR1 KO splenocytes produced less IL-4 compared to WT splenocytes. Hence, mSIGNR1 KO mice have a more Th1 dominated immune response when compared to WT mice, suggesting a role for mSIGNR1 in the Th1/Th2 balance of the immune response against *M. tuberculosis*. However, the increased IFNγ production by splenocytes early in infection did not result in an enhancement of antibacterial response by mSIGNR1

KO mice.

mSIGNR1 could be involved in the binding and internalization of *M. tuberculosis* for degradation, since mSIGNR1 is a receptor for *M. tuberculosis*. However, mSIGNR1 is not expressed by alveolar macrophages and we did not observe any induction of mSIGNR1 expression by alveolar macrophages or influx of mSIGNR1 positive cells during infection, suggesting that mSIGNR1 is not involved in the initial or late capture of *M. tuberculosis* in the lung. Indeed, no differences were observed in the outgrowth of the bacteria and local levels of IL-10, IFNγ or IL-4.

Recent studies have demonstrated that mSIGNR1 is involved in the early defense against *S. pneumoniae*[21,22]. Lanoue *et al.* demonstrated that, upon intraperitoneal infection of *S. pneumoniae*, mSIGNR1 expressed by peritoneal macrophages enhanced clearance of *S. pneumoniae* by phagocytosis[21]. Recently, we have demonstrated that upon intranasal infection with *S. pneumoniae*, mSIGNR1 KO mice did not clear bacteria from lung and blood, and displayed severely enhanced inflammatory parameters compared to the WT mice. In addition, the levels of anti-phosphorylcholine IgM did not increase during infection in mSIGNR1 KO mice whereas the antibody level did increase during infection in the WT mice. These antibodies are essential for protection against infection with *S. pneumoniae*. Hence, mSIGNR1 seems to play a role in the early antibody-mediated immune response against *S. pneumoniae*. Host defense against intracellular *M. tuberculosis* infection mainly depends on cell-mediated immunity[1,28]. Although in the early phase of infection, mSIGNR1 influences the Th1/Th2 balance of the immune response evoked by *M. tuberculosis*, this shift towards a Th1 response was not enough to protect against infection. Importantly, mSIGNR1 is not expressed by alveolar macrophages even after infection, which can explain the minor role of mSIGNR1 in the pathogenesis of *M. tuberculosis*.

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Chapter 5

DC-SIGN specifically recognizes *Streptococcus pneumoniae* serotypes 3 and 14

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Abstract

The Gram-positive bacterium *Streptococcus pneumoniae* is the leading causative pathogen in community-acquired pneumonia. The ever-increasing frequency of antibiotic-resistant *S. pneumoniae* strains severely hampers effective treatments. Thus, a better understanding of the mechanisms involved in the pathogenesis of pneumococcal disease is needed; in particular of the initial interactions that take place between the host and the bacterium. Recognition of pathogens by dendritic cells is one of the most crucial steps in the induction of an immune response. For efficient pathogen recognition, dendritic cells express various kinds of receptors, including the dendritic cell-specific C-type lectin DC-SIGN. Pathogens such as *Mycobacterium tuberculosis* and HIV target DC-SIGN to escape immunity. Here the in vitro binding of DC-SIGN with *S. pneumoniae* was investigated. DC-SIGN specifically interacts with *S. pneumoniae* serotype 3 and 14 in contrast to other serotypes such as 19F. While the data described here suggest that DC-SIGN interacts with *S. pneumoniae* serotype 14 through a ligand expressed by the capsular polysaccharide, the binding to *S. pneumoniae* serotype 3 appears to depend on an as yet unidentified ligand. Despite the binding capacity of the capsular polysaccharide of *S. pneumoniae* 14 to DC-SIGN, no immunomodulatory effects on the dendritic cells were observed. The immunological consequences of the serotype-specific capacity to interact with DC-SIGN should be further explored and might result in new insights in the development of new and more potent vaccines.

5.1 Introduction

Streptococcus pneumoniae (pneumococcus) is a major cause of bacterial pneumonia, otitis media, bacteremia and meningitis and is a leading cause of morbitity and mortality worldwide [1,2]. Pneumococcus is surrounded by a polysaccharide (PS) capsule which protects it from phagocytosis and is pivotal for virulence since non-encapsulated pneumococci are avirulent [3,4]. Capsular PS are known to mediate a protective humoral immune response. Based on the unique antigenic determinants of the capsular PS, pneumococcus can be defined into 90 different serotypes. Strikingly, antibodies that are protective against one serotype do not necessarily provide protection against another. Furthermore, the individual serotypes differ in virulence and immunogenicity [5,6]. Besides the PS capsule, all pneumococcal serotypes have a cell wall containing polysaccharides (cell wall polysaccharides, CWPS), which are common to all pneumococci. Unlike capsular PS, the structure of CWPS is more conserved.

Although major advances have been made in the development of pneumococcal vaccines, their efficacy is undermined by the serotype variability and genome plasticity of the pneumococcus. Moreover, the ever-increasing frequency of antibiotic-resistant *S. pneumoniae* strains severely hampers effective treatments [7]. Thus, a better understanding of

the mechanisms involved in the pathogenesis of pneumococcal disease is needed; in particular of the initial interactions that take place between the host and the bacterium.

Dendritic cells (DC) are professional antigen presenting cells that are pivotal in orchestrating an effective immune response. In order to interact with pathogens, DC express various kinds of receptors, including C-type lectins, that bind to carbohydrate structures. Dendritic cell-specific ICAM-3 grabbing non-integrin (DC-SIGN) is a type II C-type lectin that functions as a pathogen receptor. DC-SIGN contains one carbohydrate recognition domain (CRD) that binds to carbohydrate structures present on the surface of many pathogens but also on self-antigens. As a pathogen receptor, DC-SIGN interacts with a plethora of pathogens, such as HIV, hepatitis C virus, *Mycobacterium tuberculosis* and *Schistosoma mansoni* [8–11]. Next to its function as a pathogen receptor, DC-SIGN binds self-proteins and establishes cellular interactions of DC with endothelial cells during DC migration, with neutrophils during inflammation and with T cells during antigen presentation [12–14].

To further elucidate the in vivo function of DC-SIGN, five murine DC-SIGN homologues have been cloned [15]. The most extensively studied is mSIGNR1, which is expressed on marginal zone macrophages (MZM) of the spleen and peritoneal macrophages [16,17]. Recently, it has been shown that mSIGNR1 is involved in the in vivo capture of different pneumococcal capsular serotypes, e.g. serotypes 3, 14, 23 and 26 (also known as serotypes 3, 14, 23F and 6B, respectively, according to the Danish nomenclature) [18]. Furthermore, mSIGNR1 was shown to be required for immunity against infection with *S. pneumoniae* serotype 2 and 14 [19]. Similarly, DC-SIGN may also be involved in the orchestration of the immune response against *S. pneumoniae*.

We studied the interaction of DC-SIGN with pneumococcal PS serotypes, commonly causing disease [20,21]. Strikingly, DC-SIGN interacted only with pneumococcal PS serotype 14. However, when the interaction with heat-killed pneumococci was studied, binding was observed to serotype 3 in addition to serotype 14. This indicates that DC-SIGN may interact with other antigenic epitopes found on serotype 3.

Moreover, we observed that *S. pneumoniae* capsular polysaccharides did not alter the activation of DC by LPS, suggesting that *S. pneumoniae* capsular polysaccharides do not target DC-SIGN to evade the immune system. Still, the immunological consequences of this serotype-specific capacity to interact with DC-SIGN should be further explored and might result into new insights in the development of new and more potent vaccines.

5.2 Materials and Methods

Antibodies, cells and bacteria

The stable Raji transfectant expressing DC-SIGN was generated by electroporation of 10 μg pRc/CMV-DC-SIGN as previously described [9]. The Raji-1 cells used were previously called THP-1 cells by mistake [22]. Immature DC were cultured from monocytes

in the presence of IL-4 and GM-CSF (500 and 800 U/ml, respectively; Schering-Plough, Brussels, Belgium)[23,24]. All experiments were performed using 5-7 days old immature DC. *S. pneumoniae* serotype 3 (ATCC 6303, Rockville, MD), 14 or 19 (CDC, Atlanta, GA), were cultured in Todd Hewitt broth containing 10% fetal bovine serum and heat-killed by incubation at 56°C for 30 minutes. Heat-killed preparations were cultured on blood agar plates at 37°C for 20 hours to confirm the absence of viable organisms.

The following antibodies were used: AZN-D1 and AZN-D2 (anti-DC-SIGN)[9]; Clone 19 (anti-MR, BD Biosciences).

Soluble DC-SIGN-Fc adhesion assay

DC-SIGN-Fc consists of the extracellular portion of DC-SIGN (amino acid residues 64-404) fused at the COOH terminus to a human IgG1-Fc fragment[25]. The soluble DC-SIGN adhesion assay was performed as follows. Soluble ligands were coated onto ELISA plates 10 μg/ml for 18 hours at 4°C followed by blocking with 1% BSA for 30 minutes at 37°C. Soluble DC-SIGN-Fc supernatant (0.75 μg/ml) was added and incubated for 2 hours at room temperature. Unbound DC-SIGN-Fc was washed away and binding was detected by peroxidase-labeled goat anti-human IgG (Jackson). Absorbance was measured at 450 nm. Specificity of binding was determined in the presence of either 20 μg/ml blocking antibodies or 10 mM EGTA.

Ligand coating of fluorescent beads

Carboxylate-modified TransFluorSpheres (Molecular Probes, Eugene, OR) 75 μl (about 3.3×10^{10}/ml) were coated with the capsular polysaccharides of *S. pneumoniae* serotype 3, 14, 19 and cell wall polysaccharide by incubation for 15 minutes at room temperature with 10 μg/ml polysaccharides of *S. pneumoniae*. 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDAC) (Sigma Chemie) dissolved (1.33 mg/ml) in MES-buffer (50 mM MES pH 6.0) was added to the solution and incubated for 2 hours. The reaction was stopped by addition of glycin to a final concentration of 100 mM for 30 minutes. The beads were washed three times with PBS and resuspended in 75 μl PBS, 0.5% BSA. This suspension remains stable for two months if stored at 4°C[26].

The gp120 coated beads were generated by incubating streptavidin-coated beads with biotinylated goat $F(ab)_2$ anti-human Fc fragments (Jackson Immunoresearch Laboratories Inc., Westgrove, PA) for 2 hours at 37°C. The beads were washed and incubated with cell supernatant containing gp120-Fc recombinant protein overnight at 4°C[9].

Adhesion of ligand-coated beads to cells was performed as described previously[26].

Binding assay

DC or Raji cells stably expressing DC-SIGN were pre-incubated in adhesion buffer (20 mM Tris- (tris(hydroxymethyl)aminomethane)-)HCl, pH 8.0, 150 mM NaCl, 1mM $CaCl_2$, 2mM $MgCl_2$, 0.5% BSA) with either 1 mg/ml mannan (*Saccharomyces cerevisiae*, SIGMA, St. Louis, MO, USA), 10 mM EGTA, 20 μg/ml AZN-D1, 20 μg/ml AZN-D2, 1×10^8 CFU/ml *Escherichia coli* (strain AMC B12G1) or 1×10^8 CFU/ml heat-killed *S. pneumoniae* serotype 3, 14 or 19F, for 15 minutes at 37°C. Gp120 beads or beads coated with pneumococcal PS serotypes 3, 14, 19F or CWPS were added and the suspension was incubated for 45 minutes at 37°C. Cells were washed with adhesion buffer and analyzed using flow cytometry (FACS Calibur, BD, San Jose, CA).

Maturation assay

Immature DC (5×10^4 cells/well) were cultured for 24 hours in a total volume of 100 μl RPMI 10% FCS and in the presence of polysaccharides of *S. pneumoniae* (50 μg/ml) and LPS (10 ng/ml) unless otherwise noted. The role of DC-SIGN was determined by preincubating immature DC with AZN-D1 or AZN-D2 (20 μg/ml) for 30 minutes at 37°C. DC maturation was determined by analyzing cell-surface expression of the co-stimulatory molecules CD83 and CD86 using phycoerythrin-conjugated antibodies, which were incubated for 30 minutes at 4°C.

Cytokines in cell supernatant were measured by sandwich ELISA. The anti-cytokine antibodies (IL-6, IL-10 and IL-12, 1.0 μg/ml, Biosource) were coated onto ELISA plates in 0.05 M sodium carbonate for 18 hours at 4°C. After washing with PBS, 0.02% Tween-20, the plates were blocked with PBS 2% milk (PBS/milk) for 1 hour. After washing, samples and standards (Biosource) were incubated for 2 hours together with cytokine detecting antibody (Biosource). Standards were used to determine the concentration of cytokine production in the samples. For detecting the cytokine complex, streptavidin poly-HRP (CLB) was added for 30 minutes at room temperature. The reaction was developed by TMB and stopped by addition of 0.8 M H_2SO_4.

5.3 Results

S. pneumoniae is a major cause of morbidity and mortality worldwide. The capsular polysaccharides are crucial for virulence of the bacterium but are also involved in the elicitation of protective humoral immunity. DC-SIGN, a C-type lectin, is known for its dual role in immunity against pathogens. As this C-type lectin is able to interact with polysaccharides, DC-SIGN may be involved in the immune response against or immune modulation by *S. pneumoniae*.

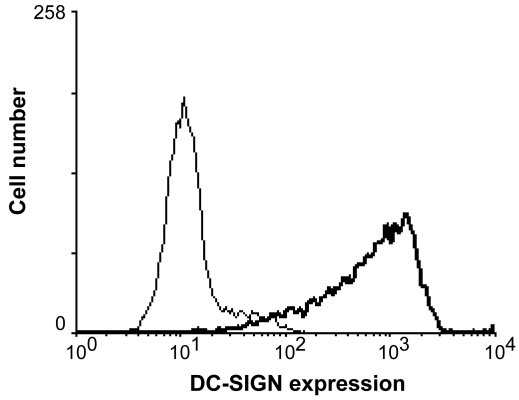

Figure 5.1: Immature DC express high levels of DC-SIGN
The thin and thick line correspond to the isotype control and specific antibody staining (AZN-D1) respectively. One representative experiment out of three is shown.

DC-SIGN binds the capsular polysaccharide of *S. pneumoniae* serotype 14

More than 90 pneumococcal serotypes have been described for pneumococcus, based on the capsular PS structures, which differ in virulence and immunogenicity [21]. We set out to investigate the binding capacity of DC-SIGN on DC (Fig. 5.1) to a panel of pneumococcal capsular PS and CWPS. The panel is a representation of the pneumococcal serotypes that are most common cause of pneumococcal disease [20,21]. Capsular PS was coated on ELISA plates and DC-SIGN-Fc binding was analyzed. Strikingly, only serotype 14 was bound by DC-SIGN and the interaction was specific as it was blocked by anti-DC-SIGN antibodies and EGTA (Fig. 5.2).

We have demonstrated that DC interact with HIV-1 envelope gp120 through DC-SIGN previously [9]. To investigate the interaction of DC-SIGN expressed on DC with pneumococcal PS, a gp120-blocking assay was used. Thus, blocking of gp120 binding to DC indicates binding of PS to DC-SIGN. Strikingly, only capsular polysaccharide derived from pneumococcal serotype 14 blocked the binding of gp120 to DC-SIGN whereas neither the other capsular polysaccharides derived from pneumococcal serotype 1, 3, 4, 6B,18C and 19F (also known as serotype 1, 3, 4, 26, 56 and 19, respectively, according to the American nomenclature) nor the CWPS demonstrated any substantial block (Fig. 5.3).

DC-SIGN binds heat-killed pneumococci serotype 3 and 14

Since DC-SIGN interacts with the capsular polysaccharide of pneumococcal serotype 14 we set out to investigate the binding capacity to heat-killed pneumococci. In addition to serotype 14, serotype 3 was analyzed as this serotype has been described to inter-

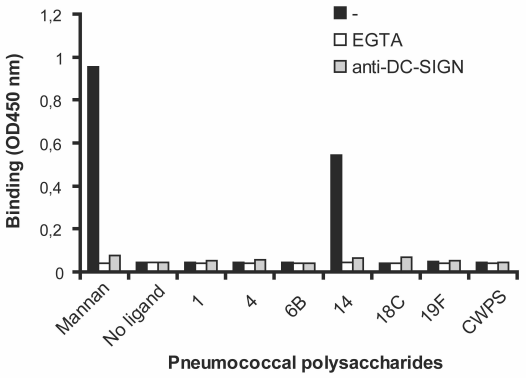

Figure 5.2: Soluble DC-SIGN-Fc specifically interacts with capsular PS serotype 14
DC-SIGN Fc binding to the capsular PS was determined by an Fc-specific ELISA. Standard deviation <0.02 OD 450. One representative experiment out of three is shown.

act with the murine homologue of DC-SIGN, mSIGNR1[18]. A gp120-blocking assay demonstrated that gp120 binding to DC-SIGN expressed on immature DC (Fig. 5.1) was not only blocked by pneumococcal serotype 14, but also by serotype 3 in contrast to *S. pneumoniae* serotype 19 (Fig. 5.4). In addition, preincubation with other bacteria such as *E. coli* did not inhibit binding of DC-SIGN to gp120 (Fig. 5.4).

The difference in binding of bacteria compared to polysaccharides of pneumococcal

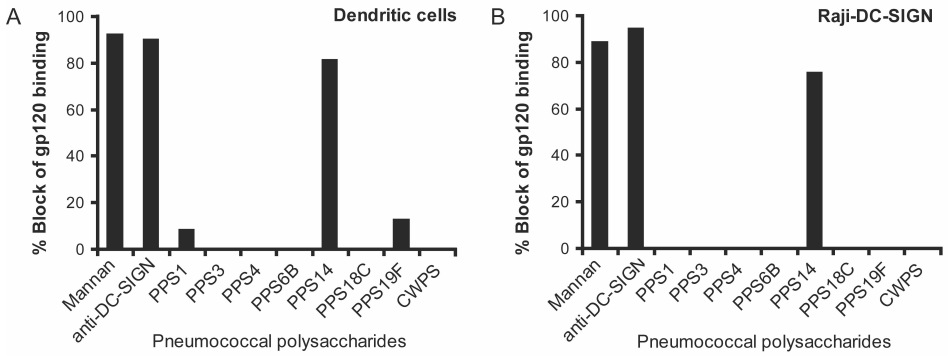

Figure 5.3: DC-SIGN specifically interacts with capsular PS serotype 14
Both DC-SIGN expressing DC and transfectants specifically bind to capsular PS serotype 14 in contrast to capsular PS serotype 1, 3, 4, 6B, 18C, 19F and CWPS. Binding of DC-SIGN on DC (A) or transfectants (B) to the different capsular PS serotypes was determined by the measurement of the inhibition of the DC-SIGN specific binding to gp120. Standard deviation for the fluorescent bead adhesion assay was <5%. One representative experiment out of three is shown.

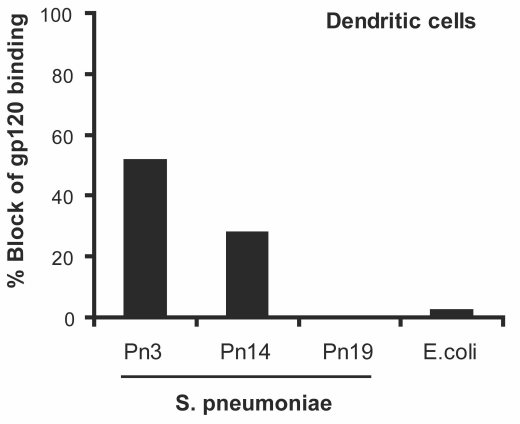

Figure 5.4: DC-SIGN specifically binds to heat-killed pneumococcal serotype 3 and 14
DC-SIGN expressing DC specifically interact with heat-killed pneumococcal serotype 3 and 14 in contrast to serotype 19. Binding of DC-SIGN on DC to three different heat-killed pneumococcal serotypes was determined by the measurement of the inhibition of the DC-SIGN specific binding to gp120. The standard deviation for the fluorescent bead adhesion assay was $<5\%$. One representative experiment out of three is shown.

serotype 3 may reflect the localization of the specific epitope recognized by DC-SIGN. In addition, the difference in binding of *S. pneumoniae* polysaccharide compared to whole bacteria could also be caused by a difference in the tertiary structure of the capsular polysaccharide. Next, fluorescent beads were coated with pneumococcal PS serotypes 3, 14, 19F and CWPS, respectively, to provide multivalence. Fluorescent beads coated with PS derived from pneumococcal serotype 14 bind DC-SIGN expressed by immature DC and DC-SIGN expressed on Raji cells. This binding to DC is DC-SIGN specific, since binding could be blocked by the DC-SIGN specific blocking antibody AZN-D1 (Fig. 5.5). Fluorescent beads coated with PS derived from pneumococcal serotypes 3, 19F or pneumococcal CWPS did not bind to DC-SIGN. In conclusion, DC-SIGN recognizes an epitope present on pneumococcal serotype 3, which is not present in the PS capsule.

Capsular polysaccharides of *S. pneumoniae* do not influence DC-maturation

Next, the functional implications of the specific binding of *S. pneumoniae* serotype 14 by DC-SIGN were investigated. Previously, it has been shown that binding of a cell wall component of *M. tuberculosis* to DC-SIGN blocks LPS-induced maturation. Specifically, maturing DC exposed to the mycobacterial component ManLAM showed lower expression of the maturation markers CD80, CD83 and CD86 and secreted increased lev-

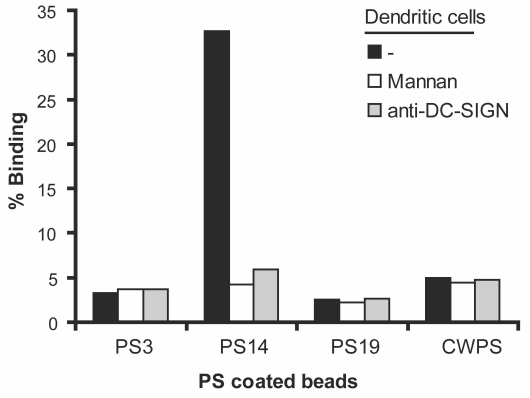

Figure 5.5: DC-SIGN specifically recognizes capsular PS serotype 14
The adhesion of DC-SIGN expressed by DC or on transfectants was determined using the fluorescent bead adhesion assay. Specificity was determined by the polysaccharide mannan and the DC-SIGN specific antibody AZN-D1. Standard deviation for the fluorescent bead adhesion assay was $<5\%$. One representative experiment out of three is shown.

els of the immunomodulatory cytokine IL-10[27]. Thus, DC-SIGN expressing immature DC were co-cultured with LPS and pneumococcal capsular polysaccharide serotype 14. However, no immunosuppressive effects such as IL-10 production by DC or a reduction in the expression of the maturation markers CD83 and CD86 by DC was observed (Fig. 5.6A and B and data not shown). Production of IL-12p40 by DC was not influenced by the capsular PS (Fig. 5.6C).

Thus, DC-SIGN displays a clear binding specificity for *S. pneumoniae* serotype 3 and 14 in contrast to serotype 19 or the polysaccharides of the cell wall. Furthermore, binding to *S. pneumoniae* serotype 3 does not seem to be mediated via the capsular polysaccharide. Overall, we demonstrate that *S. pneumoniae* binding to DC-SIGN is serotype dependent. However, the functional implications of the serotype specificity of DC-SIGN remain to be elucidated.

5.4 Discussion

S. pneumoniae still poses a great threat to human health. Effective vaccines are still needed and the emergence of antibiotic resistant pneumococci is a large drawback in the battle against this pathogen. In this study the interaction between *S. pneumoniae* and the type II C-type lectin DC-SIGN was investigated. Interaction of this bacterium with DC that express DC-SIGN could give us more insight into the early events of *S. pneumoniae* infection. DC-SIGN, via its cross talk with T cells and neutrophils, is able to enhance immune activation. In contrast, DC-SIGN has also been described as a target for

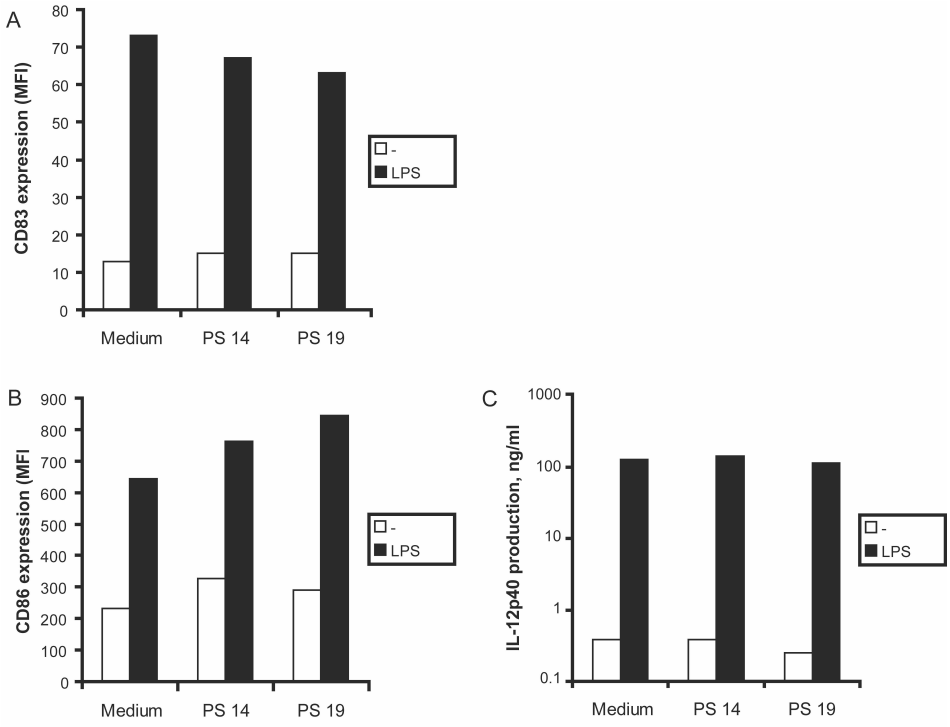

Figure 5.6: Capsular PS serotype 14 binding to DC does not result in immune modulation
Capsular PS serotype 14 and 19 do not induce activation of immature DC. LPS-induced activation of DC is not blocked by capsular PS serotype 14 or 19. Immature DC were incubated with capsular PS serotype 14 and 19 for 24 hours and activation was determined by measuring the expression of CD83 (A) and CD86 (B). (C) Supernatants were harvested after 24 hours and the IL-12 production was measured by ELISA. One representative experiment out of three is shown. Standard deviation $<$0.02 OD 450.

pathogens to escape immunity [11,27].

This report demonstrates that DC-SIGN specifically binds capsular polysaccharide serotype 14 in contrast to the other important capsular PS tested (serotypes 1,3, 4, 6B, 18C, 19F) and pneumococcal CWPS. However, DC-SIGN expressed on human immature DC also interacts with encapsulated *S. pneumoniae* serotype 3 in addition to serotype 14. The epitope bound by DC-SIGN on encapsulated *S. pneumoniae* serotype 3 was not detected in the PS capsule, but probably involves another unidentified antigen [28]. Another explanation could be that the epitope recognized by DC-SIGN is initially present on the capsule of pneumococcal serotype 3, but is destroyed upon extraction of the polysaccharide fraction. This is in concurrence with the fact that upon removal of the capsule, mSIGNR1 no longer binds pneumococcal serotype 3 [18]. However, in that specific exper-

iment the bacteria were heated at 95°C, which likely destroys bacterial surface proteins. Furthermore, Kang *et al.* have shown that *S. pneumoniae* serotype 3 capsular polysaccharide is able to block dextran-FITC binding to mSIGNR1, the murine homologue of DC-SIGN, although to a lesser extent than serotype 14[18]. This is in contrast to our data that identify only capsular polysaccharide 14 and not capsular polysaccharide of serotype 3 as a ligand for DC-SIGN, suggesting a difference in binding specificity between the two homologues. However, this difference in binding might also be reflecting a lower binding affinity of DC-SIGN for pneumococcal serotype 3 capsular PS.

Pneumococcal serotype 14 is a leading cause of invasive pneumococcal disease in the US and Western Europe[20,29]. Strikingly, of a panel of capsular polysaccharides derived from the most common *S. pneumoniae* serotypes causing disease, only the capsular polysaccharide of *S. pneumoniae* serotype 14 is bound by DC-SIGN. Recent studies have shown that pathogens target DC-SIGN to evade the immune system. For example, *M. tuberculosis* is able to down-regulate DC maturation by the ligation of its cell wall component ManLAM to DC-SIGN on DC[27]. Possibly, the capacity of *S. pneumoniae* serotype 14 to bind DC-SIGN induces suppression of the immune response against this pathogen, facilitating the large contribution of this pneumococcal serotype to pneumococcal disease. To investigate this, DC-SIGN expressing immature DC were co-cultured with LPS and pneumococcal capsular polysaccharide serotype 14. However, no immunosuppressive effects such as a down-regulation of IL-12 production by DC or a down-regulation of the maturation markers CD80 and CD86 were observed. Possibly, *S. pneumoniae* serotype 14 uses DC-SIGN for dissemination, hereby explaining the invasive nature of this bacterium.

Thus, this study demonstrates that DC-SIGN specifically binds to *S. pneumoniae* serotype 3 and 14 whereas no binding was observed to *S. pneumoniae* serotype 19. Further experiments are needed to demonstrate the physiological relevance of the binding specificity of mSIGNR1 and DC-SIGN to different *S. pneumoniae* serotypes.

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Chapter 6

Murine specific ICAM-3 grabbing nonintegrin-related 1 (mSIGNR1) expressed by marginal zone macrophages is essential for defense against pulmonary *Streptococcus pneumoniae* infection

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Abstract

The dendritic cell-specific ICAM-3-grabbing nonintegrin (DC-SIGN) homologue, murine SIGN-related 1 (mSIGNR1) is a pathogen receptor expressed by splenic marginal zone and peritoneal macrophages, and is essential for clearance of *Streptococcus pneumoniae* by phagocytosis after intraperitoneal infection. Here, we identified an important in vivo function for mSIGNR1 in *S. pneumonia* infection induced via its natural entrance route. Upon intranasal infection with *S. pneumoniae*, mSIGNR1-deficient mice did not clear bacteria from lung and blood, and displayed severely enhanced inflammatory parameters compared to the wild-type mice. However, mSIGNR1 is not expressed by alveolar macrophages, suggesting that another mechanism than a decrease in phagocytosis is responsible for this difference. Natural anti-phosphorylcholine IgM produced by marginal zone B cells is essential for protection against infection with *S. pneumoniae*. Strikingly, during infection, mSIGNR1-deficient mice failed to produce a rapid anti-phosphorylcholine IgM response. Marginal zone macrophages have been suggested to capture antigens for presentation to marginal zone B cells. We demonstrate that marginal zone macrophages from mSIGNR1-deficient mice in contrast to wild-type mice are not able to capture pneumococci from blood, suggesting that mSIGNR1 on marginal zone macrophages captures *S. pneumoniae* for antigen presentation to and activation of marginal zone B cells, resulting in an anti-phosphorylcholine IgM response.

6.1 Introduction

The first line of defense against invading *Streptococcus pneumoniae* is provided by the innate immune system. There is strong evidence that the complement system is important in pneumococcal infection [1], and the classical pathway, partially mediated by the binding of natural IgM antibodies to bacteria, is the most important pathway for activation of this system during innate immunity to *S. pneumoniae* [2].

Rapid antibody responses to pneumococci are also essential for effective elimination of the pathogen and, in this context, the spleen, with its highly specialized lymphoid compartment, plays a central role in clearing blood-borne pathogens. Pneumococcal polysaccharides are T-cell independent type 2 (TI-II) antigens and splenic marginal zone B cells play an important role in such responses [3,4]. Splenic marginal zone B cells join B1 B cells to generate the initial humoral IgM response in the initial 3 days of a primary immune response to particulate bacterial antigens [5].

A role in the defense against pneumococcal infection may also exist for marginal zone macrophages that have been shown to capture and concentrate both TI-II and particulate antigens circulating in the blood [6,7]. Encapsulated *S. pneumoniae* injected intravenously were rapidly captured by marginal zone macrophages [7]. The dendritic cell-specific ICAM-3-grabbing nonintegrin (DC-SIGN) homologue, murine SIGN-related 1

(mSIGNR1) is a C-type lectin involved in the capture of TI-II antigens by marginal zone macrophages, since the rapid capture of pathogen polysaccharides, such as mannan and dextran, by marginal zone macrophages upon intravenous injection is inhibited by antibodies against mSIGNR1[8,9]. Moreover, using blocking antibodies Kang *et al.* demonstrated that mSIGNR1 is involved in the capture of different serotypes of capsular polysaccharides from *S. pneumoniae*[10]. Recently, Lanoue *et al.* demonstrated that mSIGNR1 plays a crucial role in the immune response against intraperitoneal infection of *S. pneumoniae* serotype 2 and 14[11]. The authors show that mSIGNR1 expressed by peritoneal macrophages plays a crucial role in binding and concomitant phagocytosis of the bacteria. However, the natural infection pathway of *S. pneumoniae* is through inhalation via the upper airways, and mSIGNR1 is not expressed by the residing alveolar macrophages. Therefore, we have investigated the role of mSIGNR1 in the defense against pulmonary infection with *S. pneumoniae* using mice with a genetic deletion of mSIGNR1 (SIGNR1$^{-/-}$)[11]. We demonstrate that SIGNR1$^{-/-}$ mice are more susceptible to pulmonary *S. pneumoniae* infection than WT mice, and that SIGNR1$^{-/-}$ mice fail to induce an early IgM response against *S. pneumoniae*. Furthermore, our data suggest that mSIGNR1 expressed by marginal zone macrophages is pivotal for the capture of *S. pneumoniae* from blood, and is involved in the induction of the early IgM response against *S. pneumoniae*, which might be necessary for protection against these bacteria.

6.2 Materials and Methods

Mice and bacteria

SIGNR1$^{-/-}$ mice were a generous gift from A. McKenzie (Cambridge, UK). C57BL/6×129 WT and SIGNR1$^{-/-}$ mice were bred in the animal facility of the VU University Medical Center under specific pathogen-free conditions, and were kept in the animal facilities of the VU University Medical Center and the Academic Medical Center in Amsterdam, The Netherlands. Age- and sex-matched mice were used in all experiments. The experiments have been approved by the Animal Care and Use Committee of the University of Amsterdam and the VU University Medical Center.

S. pneumoniae serotype 3 (ATCC 6303, Rockville, MD) were used in mid-logarithmic phase and concentrations were determined by plating dilutions on sheep-blood agar plates.

Immunofluorescence analysis

Cryosections, 8 μm thick, were fixed in dehydrated acetone for 10 minutes, rehydrated with PBS and stained for 90 minutes at 37°C with primary antibodies ED31 (anti-MARCO), SER-4 (anti-Sialoadhesin) or ERTR-9 (anti-SIGNR1). The appropriate secondary antibodies were used for detection. Heat-killed *S. pneumoniae* serotype 3 was detected by a pneumococcal polysaccharide serotype 3-specific rabbit polyclonal (Statens

Serum Institute, Copenhagen, Denmark).

In vivo antigen capture by marginal zone macrophages

Naive mice were injected intravenously into the tail veins with either 100 μl FITC-dextran (0.5 mg/ml, 500-kDa dextran-FITC; Molecular Probes, Eugene, OR), or 200 μl heat killed *S. pneumoniae* serotype 3 (1.3×10^6 CFU/ml). After 45 minutes mice were sacrificed and spleens were isolated and antigens were detected by immunofluorescence analysis.

Binding Assay

Raji-1 cells stably expressing mSIGNR1 or freshly isolated peritoneal macrophages were preincubated in adhesion buffer (20 mM Tris, pH 8.0, 150 mM NaCl, 1 mM $CaCl_2$, 2 mM $MgCl_2$, 0.5% BSA) with either 2 mg/ml mannan (SIGMA, St. Louis, MO), 50 mM EGTA, 2.6×10^7 CFU/ml heat killed *S. pneumoniae* serotype 3 (ATCC 6303, Rockville, MD), 1×10^9 CFU/ml *Escherichia coli* (strain AMC B12G1) or 2×10^9 CFU/ml heat-killed non-typeable *Haemophilus influenzae* (strain 12 kindly donated by S. J. Barenkamp, St. Louis, MO) for 15 minutes at room temperature. Dextran-FITC (50 μg/ml, 500 kDa; Molecular Probes, Eugene, OR) was added and after 45 minutes at 37°C analyzed by flow cytometry.

Induction of pneumonia

Pneumonia was induced as described previously [12]. Mice were inoculated with bacteria in a 50 μl volume (5×10^4 CFU) intranasally, and 24 or 48 hours after infection, mice were sacrificed. CFU were determined from tissue homogenates.

Cytokines and chemokines were measured using specific ELISA (R&D Systems, Minneapolis, MN) according to the manufacturers instructions. Lungs for histology were harvested at 24 and 48 hours after infection, fixed in 10% buffered formalin in PBS for 24 hours and embedded in paraffin. Lung sections, 4 μm thick, were stained with hematoxylin and eosin, and scored by a pathologist who was blinded for groups [13]. Lung inflammation and damage were scored by analyzing the entire lung surface with respect to the following parameters: interstitial inflammation, oedema, endothelialitis, bronchitis and pleuritis. Each parameter was graded on a scale of 0-5; from 0: absent to 5: severe. The total "lung inflammation score" was expressed as the sum of the scores for each parameter, the maximum being 25.

Plasma samples were analyzed for the presence of anti-PC IgM antibodies by ELISA on PC-BSA-coated wells. Specificity was determined by subtracting the coating buffer background levels from the PC coated values. All data are expressed as mean $\pm$ SEM unless indicated otherwise. Comparisons between groups were performed with Mann-

Whitney *U* tests using GraphPad Prism version 3.00, GraphPad Software (San Diego, CA). Values of $P<0.05$ were considered statistically significant.

Immunization

Naive animals were injected intraperitoneally with 0.1 mg/ml trinitrophenol-keyhole limpet hemocyanin (TNP-KLH) in 0.5 ml PBS. Sera were collected before and weekly after injection for assessment of TNP-KLH specific IgG antibodies by ELISA.

6.3 Results

mSIGNR1 expression and function

The C-type lectin mSIGNR1 is expressed by splenic marginal zone macrophages and peritoneal macrophages[8,14], but not by alveolar macrophages in the lung (data not shown). Initially, the lack of mSIGNR1 expression by marginal zone and peritoneal macrophages of SIGNR1$^{-/-}$ mice was confirmed (Fig. 6.1A and B). mSIGNR1 deficiency in marginal zone macrophages from SIGNR1$^{-/-}$ mice did not alter the architecture of the macrophage subsets present in the marginal zone (Fig. 6.1A), since the localization of both the MARCO^{+} marginal zone macrophages and the sialoadhesin^{+} marginal zone metallophilic macrophages were comparable to WT (Fig. 6.1A). An intravenous injection of the TI-II antigen dextran-FITC results in a rapid capture by marginal zone macrophages through mSIGNR1[8]. As expected, the marginal zone macrophages from SIGNR1$^{-/-}$ mice failed to capture dextran-FITC after intravenous administration (Fig. 6.1A). Similarly, mSIGNR1^{+} peritoneal macrophages efficiently bound dextran-FITC, whereas peritoneal macrophages from SIGNR1$^{-/-}$ mice did not (Fig. 6.1C). These data support an essential role for mSIGNR1 in the capture of TI-II antigens by both marginal zone and peritoneal macrophages.

mSIGNR1 interacts with *S. pneumoniae* serotype 3

To investigate the role of mSIGNR1 in infection with the virulent *S. pneumoniae* serotype 3, the mSIGNR1 interaction with this pneumococcus was studied using a dextran-blocking assay. Cell lines expressing mSIGNR1 efficiently capture dextran-FITC, and this interaction was blocked by pre-incubation of the transfectants with *S. pneumoniae* serotype 3 (Fig. 6.2A). In contrast, pre-incubation with other bacteria such as *Escherichia coli* and non-typeable *Haemophilus influenzae* did not inhibit binding of mSIGNR1 transfectants to dextran-FITC (Fig. 6.2A). Thus, while mSIGNR1 binds whole *S. pneumoniae* serotype 3, it does not interact with other bacteria such as *E. coli* and *H. influenzae* (Fig. 6.2A).

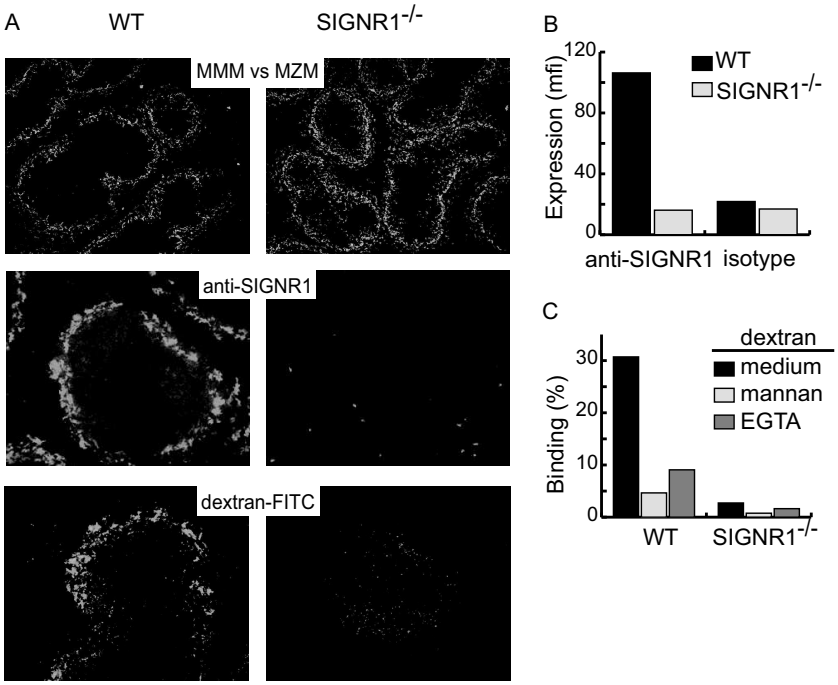

Figure 6.1: mSIGNR1 captures blood-borne TI-II antigens in vivo
(color reprint: Fig. A.3, pp. 156)
(A) The splenic marginal zone from SIGNR1$^{-/-}$ mice contains both marginal zone macrophage subsets (marginal zone macrophages (MZM) and marginal metallophilic macrophages (MMM) respectively), but does not capture TI-II antigens. Spleens from naive WT (left) and SIGNR1$^{-/-}$ (right) mice were stained for MARCO^{+} MZM in red and SER-4^{+} MMM in green (upper panel), for mSIGNR1 with ERTR-9 (middle panel) and for capture of dextran-FITC 45 minutes after intravenous administration (lower panel). These data are representative for three animals of both genotypes. (B and C) Peritoneal macrophages from SIGNR1$^{-/-}$ mice do not capture TI-II antigens. Peritoneal macrophages were analyzed for mSIGNR1 expression using ERTR-9 (B), and binding to dextran-FITC was analyzed in the presence and absence of the blocking reagents mannan and EGTA (C). These data are representative for three independent experiments.

SIGNR1$^{-/-}$ mice are more susceptible to pulmonary infection with *S. pneumoniae* serotype 3

Next, WT and SIGNR1$^{-/-}$ mice were challenged by intranasal administration of 5×10^4 CFU *S. pneumoniae* serotype 3. To investigate the role of mSIGNR1 during infection of *S. pneumoniae*, we determined the bacterial load in the lungs 24 and 48 hours after infection. Between 24 and 48 hours after infection, the bacterial load in SIGNR1$^{-/-}$ mice increased, whereas the WT mice started clearing the bacteria (Fig. 6.2B). Thus,

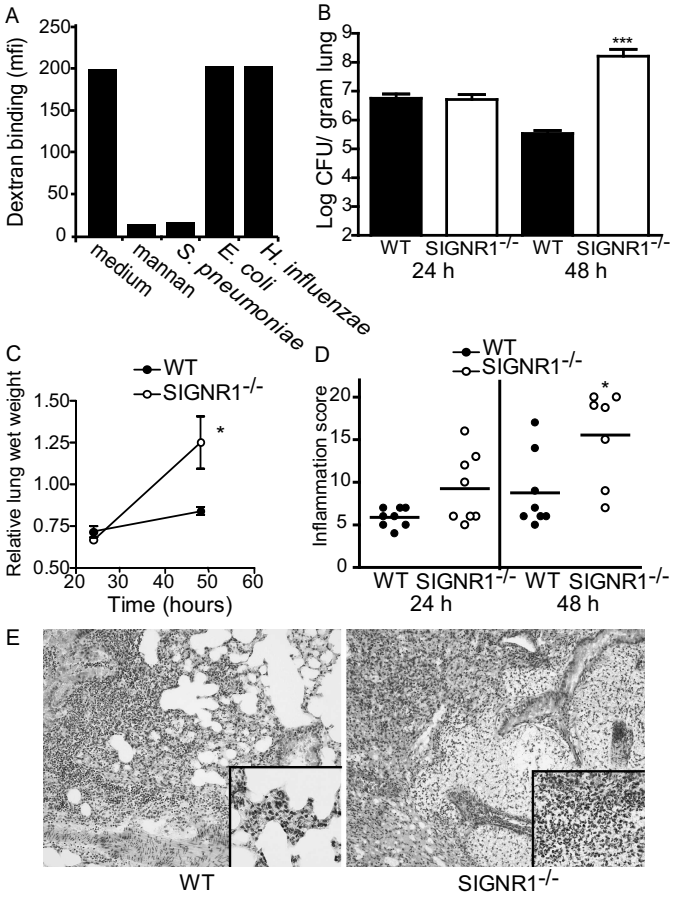

Figure 6.2: SIGNR1$^{-/-}$ mice fail to efficiently clear *S. pneumoniae* from the lungs despite the induction of a strong inflammatory response in vivo

(A) mSIGNR1 interacts with *S. pneumoniae* serotype 3. mSIGNR1 transfectants were incubated with dextran-FITC and the ability of different bacteria to inhibit this interaction was measured. (B and C) *S. pneumoniae* are not cleared from the lungs of SIGNR1$^{-/-}$ mice. WT and SIGNR1$^{-/-}$ mice were inoculated with 5×10^4 *S. pneumoniae* and bacterial outgrowth in lungs (B) and relative lung wet weight (weight lung/total mouse weight, C) was determined. (D) SIGNR1$^{-/-}$ mice have a more severe lung inflammation and damage than WT mice due to *S. pneumoniae* infection. Lung inflammation and damage were scored with respect to the following parameters: interstitial inflammation, oedema, endothelialitis, bronchitis and pleuritis. Each parameter was graded on a scale of 0-5; from 0: absent to 5: severe. The total "lung inflammation score" was expressed as the sum of the scores for each parameter, the maximum being 25. Horizontal bars represent mean inflammation scores. (E) Neutrophil influx is increased in lungs from SIGNR1$^{-/-}$ mice due to *S. pneumoniae* infection. Representative slides of lung histopathology; original magnification $\times$10; insert magnification $\times$40. *P $<$0.05, ***P$<$0.001.

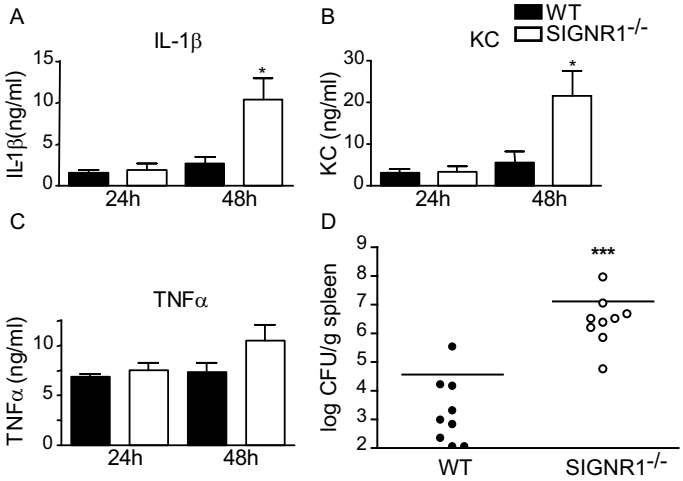

Figure 6.3: Upon infection with *S. pneumoniae*, SIGNR1$^{-/-}$ mice display similar early pulmonary cytokine responses compared to WT mice

Cytokine production in the lung (A, B and C) was measured 24 and 48 hours after infection. Bacterial outgrowth from the spleen (D) was measured 48 hours after infection. $^{*}P < 0.05$, $^{***}P < 0.001$.

SIGNR1$^{-/-}$ mice are unable to clear the pneumococci, suggesting that mSIGNR1 is involved in the early defense against *S. pneumoniae* infection.

Clearance of bacteria from the respiratory tract during pneumococcal pneumonia is strongly dependent on the efficacy in mounting a local inflammatory response. To investigate the role of mSIGNR1 in the inflammatory response, we evaluated the inflammation of the lung during infection with *S. pneumoniae* in vivo after 24 and 48 hours. Relative lung wet weights were calculated and lung histology sections were scored as described in Section 6.2. Lungs of SIGNR1$^{-/-}$ mice were enlarged, and SIGNR1$^{-/-}$ mice displayed a significantly increased inflammation of the lungs when compared to WT (Fig. 6.2C and D). In accordance, lungs from SIGNR1$^{-/-}$ mice contained more neutrophils than those from WT and substantial more oedema, as demonstrated by hematoxylin and eosin staining and increased wet lung weight (Fig. 6.2E and C). Thus, *S. pneumoniae* induced a strong inflammatory response in SIGNR1$^{-/-}$ mice, which was associated with an increased influx of neutrophils into the lungs.

Next, we investigated whether SIGNR1$^{-/-}$ mice have different pulmonary cytokine responses to respiratory tract infection with pneumococci. Therefore, we determined the concentrations of the pro-inflammatory cytokines IL-1β, TNFα and the chemokine KC at 24 and 48 hours after infection. SIGNR1$^{-/-}$ mice did not exhibit an increased immune response early after infection, since cytokine levels did not differ between SIGNR1$^{-/-}$ and WT mice after 24 hours of infection (Fig. 6.3A, B and C). However, at 48 hours of

infection, cytokine levels in the lungs from SIGNR1$^{-/-}$ mice were strongly elevated due to massive inflammation (Fig. 6.3A, B and C).

Furthermore, five of eight SIGNR1$^{-/-}$ mice developed bacteremia after 24 hours, and seven of eight after 48 hours, compared to three of eight WT mice after both 24 and 48 hours. Dissemination of bacteria to spleen and subsequent growth was strongly increased in SIGNR1$^{-/-}$ mice, since they had 100-fold more CFU in spleen after 48 hours than did WT mice (Fig. 6.3D). These data demonstrate that, although SIGNR1$^{-/-}$ mice are able to mount a neutrophil-attracting inflammatory response upon infection with *S. pneumoniae*, the pneumococci are not cleared from the lung. Moreover, SIGNR1$^{-/-}$ mice are not able to clear the bacteria from blood, suggesting that mSIGNR1 is necessary for clearance of blood-borne pathogens.

mSIGNR1 is essential for the capture of blood-borne *S. pneumoniae* serotype 3

Although mSIGNR1 is not expressed by resting alveolar macrophages in the lung, we investigated whether mSIGNR1 is upregulated after infection with *S. pneumoniae*. Immunofluorescence analysis of lung tissue demonstrated that mSIGNR1 expression was not induced upon *S. pneumoniae* infection (data not shown), indicating that the failure of the SIGNR1$^{-/-}$ mice to clear the *S. pneumoniae* is not due to an altered function of alveolar macrophages. This was supported by a similar cytokine profile compared to WT after 24 hours (Fig. 6.3A). To further elucidate the role of mSIGNR1 in the clearance of *S. pneumoniae*, we studied the capture of *S. pneumoniae* by marginal zone macrophages. After intravenous injection, *S. pneumoniae* were captured by the marginal zone in the WT, whereas hardly any *S. pneumoniae* were detected in the marginal zone of SIGNR1$^{-/-}$ mice (Fig. 6.4A and B). Moreover, *S. pneumoniae* particles that were observed in the spleen of the SIGNR1$^{-/-}$ mice were primarily localized in the red pulp (Fig. 6.4C). Thus, mSIGNR1 is essential for the capture of whole *S. pneumoniae* by the marginal zone of the spleen.

mSIGNR1 is pivotal for the induction of a natural IgM response against *S. pneumoniae*

Splenic marginal zone B cells are essential for the rapid antibody response against TI-II polysaccharides, such as the capsular polysaccharides of *S. pneumoniae* [15]. In the early phase of pneumococcal infection, IgM antibodies, in particular natural antibodies against the *S. pneumoniae*-epitope phosphorylcholine (PC), play an important role in the protection against pneumococci [16–19]. As mSIGNR1 is able to capture *S. pneumoniae* from blood, resulting in *S. pneumoniae* accumulation in the marginal zone, close to marginal zone B cells, we investigated whether mSIGNR1 is involved in the rapid increase of natural antibody production by marginal zone B cells. Therefore, we determined the anti-PC IgM response during infection. Strikingly, whereas in WT mice natural anti-PC IgM lev-

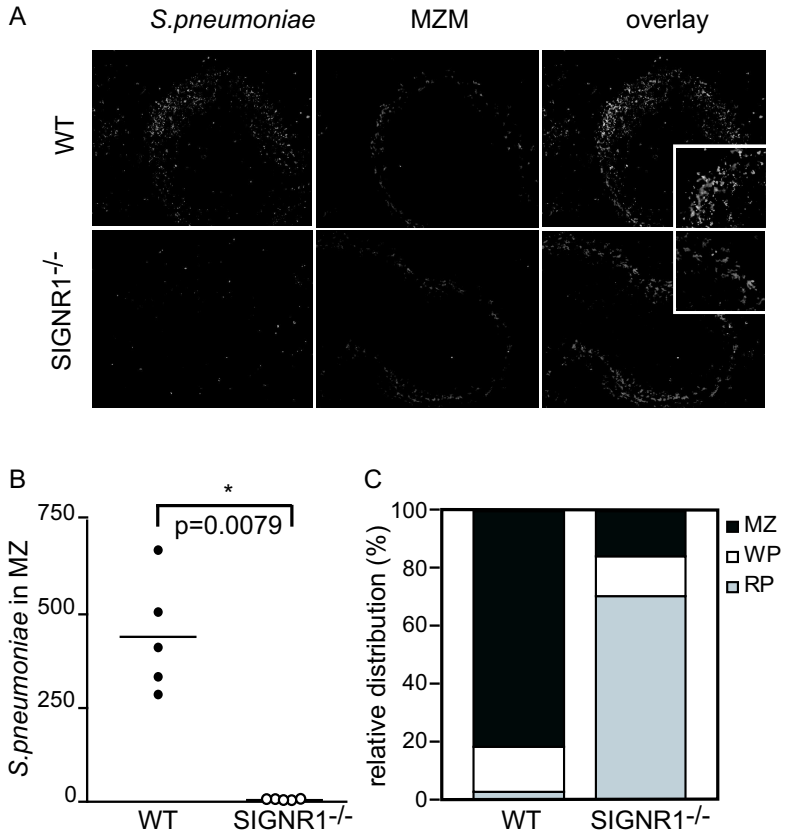

Figure 6.4: mSIGNR1 is pivotal for the in vivo capture of blood-borne *S. pneumoniae* serotype 3 by the marginal zone

(color reprint: Fig. A.4, pp. 157)

(A) *S. pneumoniae* is not captured in vivo by the splenic marginal zone in SIGNR1$^{-/-}$ mice. The capture of intravenous injected *S. pneumoniae* was determined in five representative visual fields of spleen sections of WT (upper) and SIGNR1$^{-/-}$ (lower panel) mice after 45 minutes. Spleens were stained for MARCO^{+} marginal zone macrophages (MZM) in red and *S. pneumoniae* in green. Inset shows pneumococci colocalizing and in close contact with WT MARCO^{+} MZM. Inset shows the absence of pneumococci in the marginal zone of SIGNR1$^{-/-}$ mice. (B) *S. pneumoniae* is captured by the marginal zone of the WT spleen whereas the pneumococci are hardly detected in the marginal zone of the SIGNR1$^{-/-}$ spleen. The amount of *S. pneumoniae* particles residing in the marginal zone of either WT or SIGNR1$^{-/-}$ mice was counted and compared. The capture of intravenously injected *S. pneumoniae* was determined in five representative visual fields of spleen sections of WT and SIGNR1$^{-/-}$ mice after 45 minutes. (C) The *S. pneumoniae* particles present in the SIGNR1$^{-/-}$ spleen reside mostly in the red pulp (RP) area of the spleen, in contrast to the *S. pneumoniae* particles present in the WT spleen which target to the marginal zone (MZ). These data are representative for three animals of both genotypes. $^{*}P < 0.05$.

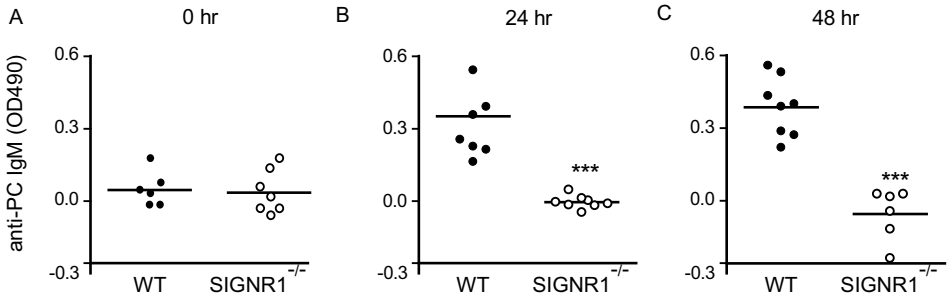

Figure 6.5: SIGNR1$^{-/-}$ mice fail to raise a rapid anti-PC IgM antibody response in vivo during infection with *S. pneumoniae*
The anti-PC IgM levels were measured in plasma before (A) and 24 (B) or 48 hours (C) after infection with 5×10^4 CFU *S. pneumoniae*. Horizontal bars represent mean anti-PC IgM levels. ***P$<$0.001.

els increased upon infection, no increase in natural anti-PC IgM production was observed during infection in SIGNR1$^{-/-}$ mice (Fig. 6.5). These data suggest that mSIGNR1-mediated capture of blood-borne *S. pneumoniae* is necessary for an efficient anti-PC IgM response by marginal zone B cells. However, upon administration of an irrelevant antigen (TNP-KLH), no differences in IgG response were observed between WT and SIGNR1$^{-/-}$ mice (Fig. 6.6). Hence, SIGNR1$^{-/-}$ mice do display a normal follicular B cell response.

6.4 Discussion

In this study we have identified an important in vivo function for the murine homologue of DC-SIGN in *S. pneumoniae* infection induced via its natural entrance route.

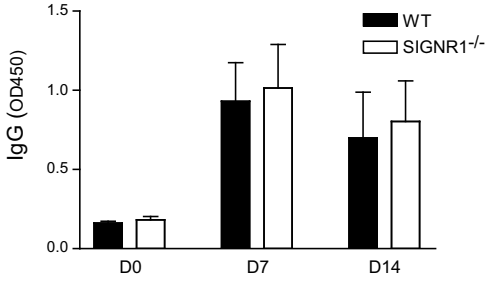

Figure 6.6: SIGNR1$^{-/-}$ mice display a normal follicular B cell-mediated immune response
The anti-TNP-KLH IgG levels were measured in serum at day 0, and at 7 and 14 days after intraperitoneal administration of 0.1 mg/ml TNP-KLH.

We demonstrate that SIGNR1$^{-/-}$ mice are more susceptible to intranasally administrated *S. pneumoniae* than WT mice. Strikingly, in contrast to infected WT mice, infected SIGNR1$^{-/-}$ mice fail to induce natural anti-PC IgM antibodies, known to be pivotal for protection against *S. pneumoniae*[16–19]. Our data suggest that mSIGNR1^{+} marginal zone macrophages are essential for the natural anti-PC IgM response by marginal zone B cells.

The marginal zone of the spleen is a unique compartment that facilitates the capture of blood-borne antigens for eliciting rapid antibody responses against bacteria[20]. Marginal zone macrophages are strategically situated in the marginal zone to capture antigens from blood. Recently, it has been demonstrated that mSIGNR1, a murine homologue of DC-SIGN, is specifically expressed by marginal zone macrophages and mediates the capture of blood-borne TI-II antigens[8,9]. Similar to DC-SIGN, mSIGNR1 interacts with different pathogens, including viruses and bacteria[10,14], suggesting that mSIGNR1 might be an important pathogen receptor on these macrophages. Marginal zone macrophages efficiently capture particulate *S. pneumoniae* present in blood[7] and our data demonstrate that mSIGNR1 mediates capture of these particulate bacteria, since marginal zone macrophages from SIGNR1$^{-/-}$ mice do not capture intravenously injected *S. pneumoniae* serotype 3 (Fig. 6.4). The failure of the SIGNR1$^{-/-}$ mice to clear the bacteria from blood after infection and the 100-fold increase in splenic bacterial load compared to WT mice supports the importance of mSIGNR1 as a pathogen receptor on marginal zone macrophages. Recently, Lanoue *et al.* demonstrated that mSIGNR1 is involved in the capture of *S. pneumoniae*[11]. In a peritonitis model using *S. pneumoniae* serotype 2 and 14, Lanoue *et al.* demonstrated that mSIGNR1 expressed by peritoneal macrophages is important in the binding and subsequent phagocytosis of the pneumococci, as peritoneal macrophages derived from SIGNR1$^{-/-}$ mice fail to clear injected *S. pneumoniae*, which causes severe infection[11]. Thus, mSIGNR1 is an important receptor for the defense against *S. pneumoniae* and is involved in the clearance of bacteria from the peritoneal cavity. However, the common route of infection by *S. pneumoniae* is through the upper airways. Strikingly, alveolar macrophages do not express mSIGNR1, suggesting that mSIGNR1 might not be involved in the defense against *S. pneumoniae* upon infection via the natural route. Therefore, we have mimicked the natural route of *S. pneumoniae* infection. In our experiments, the SIGNR1$^{-/-}$ mice displayed a significantly increased inflammation of the lungs when compared to WT mice, characterized by extensive infiltration with neutrophils and substantial more oedema (Fig. 6.2). Alveolar macrophages do not express mSIGNR1, and we did not observe mSIGNR1 expression after infection (data not shown), indicating that mSIGNR1 is not directly involved as a pathogen receptor on alveolar macrophages in the clearance of the infection. The increased influx of neutrophils in the SIGNR1$^{-/-}$ mice suggested that neutrophils were unable to clear the bacteria even though the SIGNR1$^{-/-}$ mice were able to mount an inflammatory response.

Interestingly, in pneumococcal infection, natural IgM antibodies against PC play an important role[16–19]. Recently Lanoue *et al.* demonstrated that the levels of anti-PC anti-

bodies do not differ between naive WT and SIGNR1$^{-/-}$ mice[11]. In this study we show that although the SIGNR1$^{-/-}$ mice display a normal follicular B cell-mediated immune response, intranasal infection of *S. pneumoniae* serotype 3 results in an elevation of anti-PC IgM antibodies during infection only in the WT mice. The SIGNR1$^{-/-}$ mice did not mount an efficient anti-PC IgM response, as no increase in anti-PC IgM was observed in the blood from SIGNR1$^{-/-}$ mice during infection. These data suggest that the inability of the SIGNR1$^{-/-}$ mice to clear the infection is due to the lack of early IgM antibodies against *S. pneumoniae*.

Marginal zone B cells are essential in the induction of a rapid IgM response against encapsulated bacteria, such as *S. pneumoniae*[15,21]. Although marginal zone macrophages are ideally localized to capture blood-borne antigens, their role in the early innate immune response is unclear. Early experiments suggested that marginal zone macrophages, due to their localization and ability to capture blood-borne antigens, are critical components of the TI-II antibody responses[3,22]. Recently, Kang *et al.* demonstrated that mSIGNR1 on marginal zone macrophages efficiently captures pneumococcal polysaccharides[10]. Marginal zone macrophages might capture polysaccharides for processing and presentation of the TI-II antigens to the marginal zone B cells. However, in vivo depletion of marginal zone macrophages did not lead to a decreased TI-II response against soluble TI-II antigens[20,23,24]. Our data suggest that the capture of whole encapsulated bacteria by mSIGNR1^{+} marginal zone macrophages is essential to induce an early immune response in vivo, which is pivotal for the protection against *S. pneumoniae*. It is possible that whole encapsulated bacteria are not efficiently recognized by marginal zone B cells during infection in vivo, and that marginal zone macrophages are necessary for capture and processing of bacteria followed by presentation of bacterial fragments to marginal zone B cells. The close localization of marginal zone macrophages and B cells suggests that either whole bacteria or antigens from processed bacteria might be presented by marginal zone macrophages to marginal zone B cells, reminiscent of the role of the human homologue of mSIGNR1 in HIV-1 transmission; HIV-1 is captured by dendritic cells through DC-SIGN and transmitted to T cells[25], suggesting that these C-type lectins are able to capture pathogens to present either whole particulate antigens or components to adjacent leukocytes.

Moreover, marginal zone macrophage-produced signals following the capture of bacteria might be necessary in the activation of marginal zone B cells, since TI-II antigens alone are not sufficient to induce proliferation of marginal zone B cells, and co-signals from mitogens present on bacteria as well as from accessory cells such as macrophages are needed to activate marginal zone B cells[26].

Thus, mSIGNR1^{+} marginal zone macrophages may be involved in natural antibody production against *S. pneumoniae*, which is pivotal for defense against *S. pneumoniae*. Further studies are needed to demonstrate whether the lack of anti-PC IgM antibodies is the only factor involved in the increased susceptibility of the SIGNR1$^{-/-}$ mice and how mSIGNR1 expressed by marginal zone macrophages is involved in the early humoral

immune response against *S. pneumoniae*, either through activation of marginal zone B cells, or by presentation of captured antigens to marginal zone B cells.

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Chapter 7

The interaction of marginal zone B cells with mSIGNR1 expressed by marginal zone macrophages is pivotal for early IgM production

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Abstract

The spleen plays a pivotal role in the immune defense against encapsulated bacteria such as *Steptococcus pneumoniae*. Splenic marginal zone macrophages express the C-type lectin mSIGNR1, which is crucial for the capture of *S. pneumoniae* from blood. In this study we demonstrate that mSIGNR1 is able to interact in vitro with the juxtaposing marginal zone B cell population, which is responsible for the production of the early IgM response against the *S. pneumoniae*-epitope phosphorylcholine. Strikingly, mSIGNR1-deficient mice display a reduction in the marginal zone B cell population. In addition, in vitro assays demonstrate a decrease in phosphorylcholine specificity in the splenic B cell population derived from mSIGNR1-deficient mice. Therefore we hypothesize that antigens are presented by mSIGNR1, expressed by marginal zone macrophages, to the developing marginal zone B cell population, thereby influencing the repertoire of this B cell population, which is pivotal for the early immune response against encapsulated bacteria such as *S. pneumoniae*.

7.1 Introduction

Specific intercellular adhesion molecule-grabbing nonintegrin receptor 1 (mSIGNR1) is one of the murine homologues of the C-type lectin dendritic cell-SIGN (DC-SIGN) [1]. mSIGNR1 and DC-SIGN bind high mannose-containing ligands such as mannose-capped lipoarabinomannan (ManLAM), a major cell wall component of *M. tuberculosis* and HIV-1 gp120 [2–5]. Previously it has been reported that mSIGNR1 is involved in the immune defense against *S. pneumoniae* [6,7]. Lanoue *et al.* described that mSIGNR1 expressed by peritoneal macrophages is involved in the uptake of pneumococci by peritoneal macrophages and thereby enhances the clearance of this pathogen [7]. Using an in vivo infection model where the bacterium was administrated via the lungs, we demonstrated that mSIGNR1-deficient mice are more susceptible to *S. pneumoniae*-mediated pathology. In addition, we demonstrated the absence of an increase in early antibodies directed against the pneumococci in the mSIGNR1-deficient mice.

In the early phase of pneumococcal infection, IgM antibodies, in particular those against the *S. pneumoniae*-epitope phosphorylcholine (PC), play an important role in the immune defense against this pathogen [8–11]. These antibodies are produced by marginal zone B cells, a unique IgM^{hi}, $CD21^{hi}$, $CD23^{lo}$, IgD^{lo}, $CD1d^{hi}$ B cell population residing in the marginal zone of the spleen in close contact to marginal zone macrophages expressing mSIGNR1 [12]. For years it has been speculated that marginal zone B cells can be activated by marginal zone macrophages or that marginal zone macrophages are able to transfer processed antigens to marginal zone B cells [13]. The activation of B cells without the help of T cells is referred to as the T-independent (TI) immune response. Antigens that evoke TI immune responses can be divided into two groups; TI-I and TI-II antigens. TI-I

antigens are polyclonal stimulators which evoke a TI immune response independent of T cells, whereas TI-II antigens display multiple repeating subunits that need additional T-cell derived factors to activate B cells [14–16]. Upon intravenous injection, TI-II antigens are captured by the marginal zone of the spleen [17,18]. Several studies have been performed to identify the role of the marginal zone macrophage in the TI-II response, using TI-II antigens such as Ficoll, PC and dextran, with contradictory results [19,20]. Here, we set out to investigate the interaction of mSIGNR1 with marginal zone B cells and the involvement of this interaction in the development of this specific B cell population. We demonstrate that mSIGNR1 is able to interact specifically with marginal zone B cells. In addition, mSIGNR1-deficient mice display a reduced marginal zone B cell population. Based on these results we conclude that mSIGNR1 interacts with marginal zone B cells and is involved in the recruitment of marginal zone B cells to the spleen or their survival in this area. Furthermore, we show that B cells derived from both mSIGNR1-deficient and wild-type (WT) mice display anti-PC specificity. However, the anti-PC response produced by WT-derived B cells was much more pronounced. In conclusion, mSIGNR1, expressed by marginal zone macrophages, interacts with marginal zone B cells and is involved in the production of early antibodies by this unique B cell population.

7.2 Materials and Methods

In vivo antigen capture by marginal zone macrophages

C57BL/6×129 WT and mSIGNR1-deficient mice were bred in the animal facility of the VU University Medical Center under specific pathogen-free conditions, and were kept in the animal facilities of the VU University Medical Center, Amsterdam, The Netherlands. Naive mice were injected intravenously into the tail veins with 200 μl PPS type 3 (0.25 mg/ml; American Type Culture Collection, Manassas, VA). After 45 minutes mice were sacrificed and spleens were isolated and PPS was detected by a PPS serotype 3-specific rabbit polyclonal (Statens Serum Institute, Copenhagen, Denmark) and analyzed by immunofluorescence analysis.

Immunohistochemistry

Spleens for (double) immunofluorescence were isolated and frozen in Tissue-Tek® (Sakura Finetek Europe BV, Zoeterwoude, The Netherlands). 8 μm cryosections were fixed in dehydrated acetone for 10 minutes, rehydrated with PBS and stained for 16 hours at 4°C with primary antibody MECA 367 (anti-MAdCAM) or ED31 (anti-MARCO), and subsequently with Alexa Fluor® 594-conjugated goat anti-rat IgG (Molecular Probes, Eugene, OR) for 30 minutes at room temperature. Prior to incubation with FITC-labeled anti-CD1d (CD1.1 Ly-38 1B1, BD Biosciences, Pharmingen, USA) the sections were incubated with 20% normal rat serum in PBS. Culture supernatant of the mAb MadCAM

was used, ED31 was purified from culture supernatant from hybridoma cells with protein G-Sepharose (Amersham Pharmacia Biotech, Uppsala, Sweden) and anti-CD1d was a kind gift from Prof.Dr. R. Schepers.

Flow cytometry and cell sorting

CHO transfectants expressing mSIGNR1 were generated by transfection with 10 μg pRc/CMV-mSIGNR1 plasmid by electroporation as previously described[2]. Expression was confirmed by FACS analysis using the antibody ERTR-9 (anti-SIGNR1)[21].

Splenocyte single cell suspensions were incubated with PBS supplemented with 20% normal goat serum before staining with biotin-conjugated rabbit anti-mouse IgM (Zymed Laboratories Inc., San Fransisco, CA) and rat anti-mouse IgD (Southern Biotechnology Associates Inc., Birmingham, USA) and subsequently with PE-conjugated streptavidin (BD Biosciences Pharmingen) and 488-conjugated anti-rat IgG (Jackson Immunoresearch, USA). Next, the samples were blocked with PBS supplemented with 50% normal rat serum before adding PerCP-labeled CD19 (BD Biosciences, Pharmingen, USA). The $CD19^{pos}$, IgM^{hi}, IgD^{low} cells were considered marginal zone B cells and $CD19^{pos}$, IgM^{hi}, IgD^{hi} cells were considered follicular B cells. Flow cytometry was performed on a FACS Calibur (BD, USA).

Splenocyte single cell suspensions derived from 3 C57Bl6 mice were stained with PE-conjugated anti-mouse CD21 and FITC-conjugated anti-mouse CD23 purchased from BD Biosciences, Pharmingen, USA. $CD21^{hi}$, $CD23^{low}$ cells were considered marginal zone B cells. $CD21^{hi}$, $CD23^{hi}$ cells were assigned follicular B cells. The sorted populations had a purity of at least 80%. Cells were sorted with a MoFlow (Dako Cytomation, Denmark).

Plate adhesion assay

CHO transfected with mSIGNR1 and parental CHO cells were seeded at 3×10^4 cells per well in a 96-well flat-bottom cell culture plate (Cellstar, Greiner Bio-one, Germany) and cultured overnight. The supernatant was removed from the monolayer and the cells were washed with TSA and were incubated with TSA, 4×10^4 sorted marginal zone B cells or sorted follicular B cells labelled with calceine-AM (25 μg/10^7 cells/ml; Molecular Probes Inc., Eugene, OR) for 45 minutes at 37°C. After vigorous washing, cells were allowed to adhere for 90 minutes at 37°C. The control plate was lysed by the addition of 50 μl lysis buffer (50 mmol/l Tris, 0.1% Triton X-100) immediately to gain the maximal and minimal fluorescent values. The test plate was carefully washed to remove non-adherent cells. The remaining adherent cells were lysed with 100 μl lysis buffer and the fluorescence was measured using the FluoStar Galaxy (BMG Labtechnologies, Germany). Results are expressed as the mean percentage of cells binding from triplicate wells i.e. cell adhesion percentage of maximal binding minus background fluorescence.

In vitro IgM production

Splenocyte single cell suspensions were allowed to adhere to a culture flask for 2 hours. The nonadherent cells were harvested and seeded in a 96-well plate. Next, the cells were stimulated with LPS (5 μg/ml, purified LPS from *H. pylori*, M. Monteiro, National Research Council, Ottawa, Canada), supernatant was harvested at the indicated time points. Total IgM and PC-specific IgM levels were determined by ELISA.

ELISA

Goat anti-mouse IgM (Jackson Immunoreasearch, USA) or PC-BSA (Biosearch Technologies Inc., Novato, CA, USA) were coated onto 96-well maxisorb plates (NUNC A/S, Roskilde, Denmark) in coating buffer (0.2 M $NaHCO_3$, pH 9.2) overnight at 4°C, followed by blocking with PBS 1% BSA (La Jolla, CA, USA) for 30 minutes at 37°C. The supernatants were added. After two hours of incubation at room temperature, PO-conjugated goat anti-mouse IgM (Nordic Immunology Laboratories, NL) was added for 30 minutes. The ELISA was developed using TMB (Sigma-Aldrich, USA) substrate and stopped with 0.8 M H_2SO_4. Absorption was measured using a Benchmark microplate reader (Biorad) at 450 nm.

7.3 Results

mSIGNR1 specifically captures *S. pneumoniae* capsular polysaccharides

In order to investigate the role of mSIGNR1 in the TI-II humoral immune response we first tested whether pneumococcal capsular polysaccharides, which are classified as TI-II antigens, target mSIGNR1 on marginal zone macrophages similar to whole pneumococci [14]. Indeed, the capsular polysaccharides of *S. pneumoniae* were efficiently captured by the marginal zone of WT mice, whereas these polysaccharides were not captured by the marginal zone of mSIGNR1-deficient mice (Fig. 7.1). Thus, besides the capture of whole *S. pneumoniae*, mSIGNR1 is also essential for the capture of *S. pneumoniae* capsular polysaccharides by the marginal zone of the spleen [6,22].

Decreased numbers of marginal zone B cells in mSIGNR1-deficient mice

Previously we have demonstrated a decreased early antibody production in mSIGNR1-deficient mice upon *S. pneumoniae* infection [6]. Possibly, mSIGNR1 is involved in the activation of marginal zone B cells. In addition, mSIGNR1 may be involved in the recruitment or survival of the marginal zone B cell population. Therefore, this specific B cell population was identified in mSIGNR1-deficient mice. Furthermore, the amount and location of the marginal zone B cell population was compared between WT and mSIGNR1-deficient mice using immunohistochemistry and flow cytometry. Marginal zone B cells,

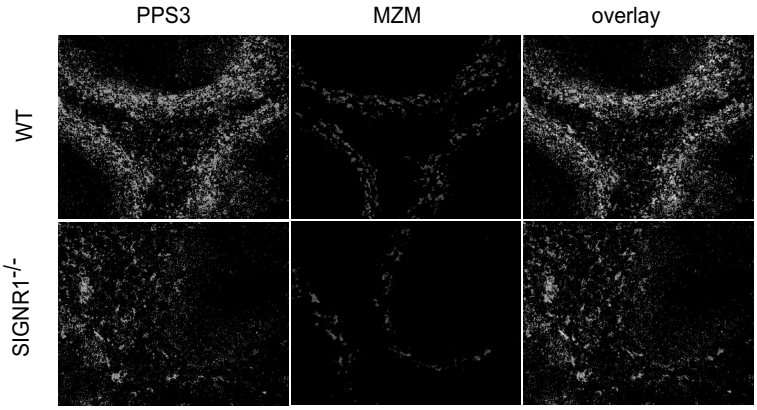

Figure 7.1: mSIGNR1 is pivotal for the in vivo capture of blood-borne *S. pneumoniae* serotype 3 polysaccharide by the marginal zone
(color reprint: Fig. A.5, pp. 158)
S. pneumoniae serotype 3 polysaccharides are retained by the marginal zone after intravenous injection in WT (upper) whereas it is dispersed throughout the spleen in mSIGNR1-deficient mice (SIGNR1$^{-/-}$, lower panel). Intravenously injected *S. pneumoniae* serotype 3 polysaccharide was detected in spleen sections of WT mice (upper) and mSIGNR1-deficient mice (lower panel) after 45 minutes. Spleens were analyzed for the expression of MARCO^{+} marginal zone macrophages (MZM) in red and pneumococcal polysaccharide serotype 3 (PPS3) in green.

identified as the CD1d-positive cells located outside the MadCAM-positive vascular endothelial cells of the marginal sinus, could be identified in both WT and mSIGNR1-deficient mice (Fig. 7.2). However, the CD1d positive population located outside the MadCAM positive rim appeared to be reduced in the mSIGNR1-deficient mice (Fig. 7.2). Similarly, the CD1d positive marginal zone B cells located in close approximation to the macrophage scavenger receptor with a collagenous structure (MARCO) positive marginal zone macrophages were also present in reduced amounts in the mSIGNR1-deficient mice (Fig. 7.2). Using flow cytometry we quantified the decreased marginal zone B cell population, identified as the IgM^{hi}IgD^{low} population, in the mSIGNR1-deficient mice (Fig. 7.3A and B).

Based on these results we conclude that in both WT and mSIGNR1-deficient mice, marginal zone B cells are present in the marginal zone of the spleen. However, the mSIGNR1-deficient mice have a reduced number of marginal zone B cells.

mSIGNR1 interacts with marginal zone B cells in contrast to follicular B cells

The decreased marginal zone B cell population in mSIGNR1-deficient mice could be explained by mSIGNR1 being involved in the homing or survival of this cell population

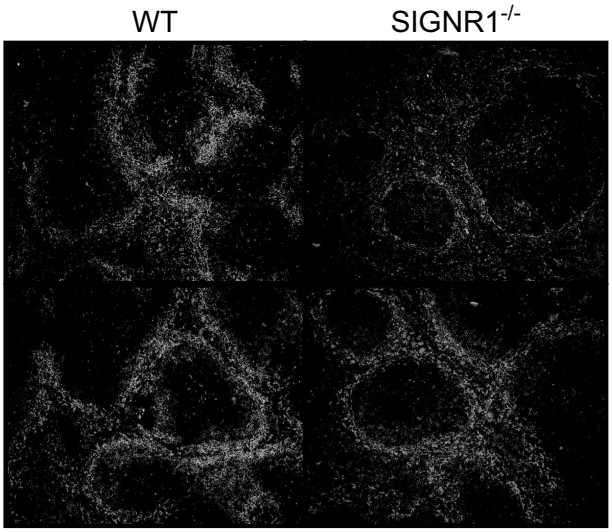

Figure 7.2: Decreased numbers of marginal zone B cells in mSIGNR1-deficient mice
(color reprint: Fig. A.6, pp. 159)
The splenic marginal zone from mSIGNR1-deficient mice contains a decreased amount of marginal zone B cells. Spleens from naive WT (left) and mSIGNR1-deficient mice (SIGNR1$^{-/-}$, right) were analyzed for the presence of MadCAM^{+} sinus-lining endothelial cells in red (upper panel) or MARCO^{+} marginal zone macrophages in red (lower panel) and the expression of CD1d in green. Marginal zone B cells were identified as the CD1d-postitive cells located outside the MadCAM^{+} rim and juxtaposed to the MARCO^{+} marginal zone macrophages. These data are representative for three animals of both genotypes.

through a direct interaction. Therefore we investigated the interaction between mSIGNR1 expressed by CHO cells (Fig. 7.4A) and marginal zone B cells. Using a plate adhesion assay we tested the adhesion of sorted marginal zone B cells and their follicular counterparts (Fig. 7.4B) to CHO cells expressing mSIGNR1. The binding of follicular B cells was similar between CHO and mSIGNR1 expressing CHO cells indicating that mSIGNR1 does not bind follicular B cells (Fig. 7.4C). However, the marginal zone B cells adhered to a larger extent to the mSIGNR1 expressing CHO cells (Fig. 7.4C). Hence, mSIGNR1 specifically binds to the marginal zone B cell population.

The B cell population derived from mSIGNR1-deficient mice displays a lower PC-specificity in vitro

We previously observed a decreased anti-PC IgM production in mSIGNR1-deficient mice upon *S. pneumoniae* infection[6]. Now that it has been established that marginal zone

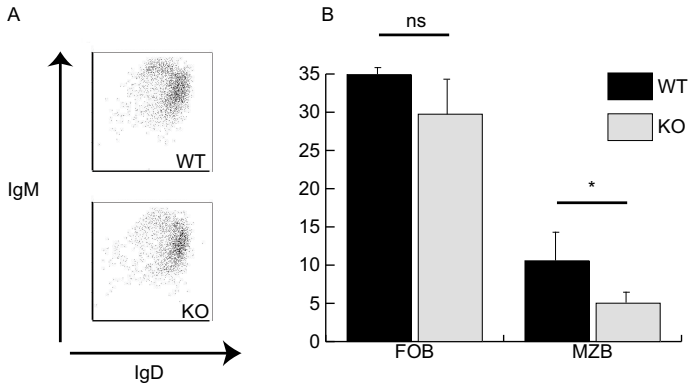

Figure 7.3: Decreased numbers of marginal zone B cells in mSIGNR1-deficient mice
Splenocytes derived from mSIGNR1-deficient mice contain less marginal zone B cells. (A) Splenocyte single cell suspensions from naive WT (A, upper panel) and mSIGNR1-deficient mice (KO, A, lower panel) were analyzed for $CD19^{pos}, IgM^{hi}, IgD^{low}$ marginal zone B cells (MZB) and $CD19^{pos}, IgM^{hi}, IgD^{hi}$ follicular B cells (FOB). (B) A comparison of the percentage of follicular (FOB) and marginal zone B cells (MZB) in the spleen between WT and mSIGNR1-deficient mice. These data are representative for three animals of both genotypes. $^{*}P < 0.05$.

B cells can interact with the juxtaposed marginal zone macrophages through mSIGNR1, we next investigated the capacity of the B cell population in the mSIGNR1-deficient mice to produce anti-PC IgM. Splenic B cells were polyclonally stimulated with LPS resulting in antibody production and both total IgM and anti-PC IgM levels were measured [23]. Strikingly, we observed that, although the B cells derived from mSIGNR1-deficient mice produce similar total IgM levels (Fig. 7.5A), there was a decreased level of anti-PC IgM produced by the mSIGNR1-deficient B cell population compared to WT B cells (Fig. 7.5B). This indicates that the splenic B cell population derived from mSIGNR1-deficient mice displays a lower anti-PC specificity compared to the WT B cell population.

7.4 Discussion

Using *S. pneumoniae* infection models, it has been described that mSIGNR1-deficient mice are more affected by this pathogen [6,7]. In both models, e.g. intraperitoneal versus intratracheal infection with *S. pneumoniae*, a higher outgrowth of bacteria and more severe pathology in the mSIGNR1-deficient mice was observed in comparison to WT mice.

Lanoue *et al.* postulated that the binding of *S. pneumoniae* to mSIGNR1 expressed by resident peritoneal macrophages leads to phagocytosis of the pathogen resulting in the clearance of the pathogen and concomitantly to less pathology in the WT mice com-

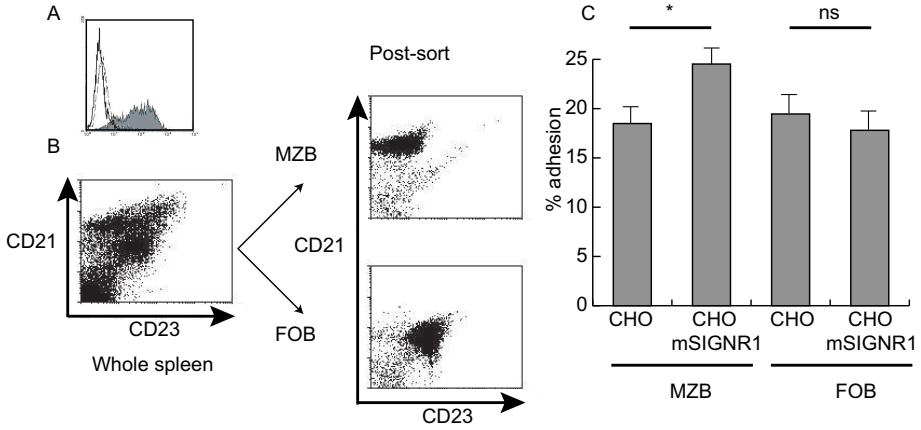

Figure 7.4: mSIGNR1 interacts with marginal zone B cells in contrast to follicular B cells
CHO-cells expressing mSIGNR1 interact with marginal zone B cells. CHO cells expressing mSIGNR1 or non-transfected parental cells (A) were analyzed for the binding of sorted marginal zone B cells (MZB) and follicular B cells (FOB) (B) using a plate adhesion assay. (A) The open histogram represents the isotype control, the dotted line represents the mock transfectant and the filled histogram indicates specific antibody staining. (C) Depicted is the percentage of specific binding relative to the maximal value. These data are representative for three independent experiments. $^{*}P < 0.05$.

pared to mSIGNR1-deficient mice that could not efficiently clear *S. pneumoniae*[7]. In the intraperitoneal infection model exerted by Lanoue *et al.* the role of phagocytosis of the pneumococci by the residing peritoneal macrophages and concomitant clearance of the pathogen dominates[7]. The role of the early humoral immune response in which antibodies are raised against PC was addressed by Lanoue *et al.*[7]. The anti-PC levels of uninfected WT and mSIGNR1-deficient mice were determined, and no difference was observed. Strikingly, the amount of anti-PC antibody in both mice strains was similar to the titer of pneumovax-immunized mice.

In our intratracheal infection study, the early anti-PC levels were monitored during infection and striking differences were found. While the anti-PC levels were raised in WT mice during the course of infection, anti-PC levels of mSIGNR1-deficient mice remained unchanged. Marginal zone B cells, which reside in close proximity to the mSIGNR1-expressing marginal zone macrophages, are able to produce these early anti-PC IgM antibodies[12].

Anti-PC IgM antibodes play an important protective role in the immune defense against *S. pneumoniae* infection[8–11]. Since the capture of *S. pneumoniae* is mediated by marginal zone macrophages and the early antibody response is mediated by marginal zone B cells we focused on the interaction between these two specialized cell populations

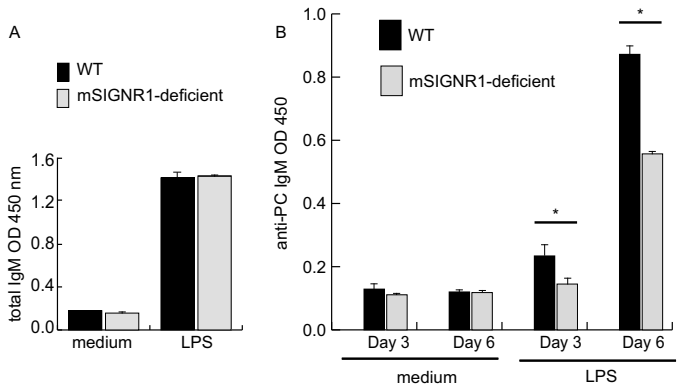

Figure 7.5: B cells derived from mSIGNR1-deficient mice produce less PC-specific IgM compared to WT derived B cells

Non-adherent splenocytes were stimulated with LPS in vitro. The production of total IgM at day 6 (A) and PC-specific IgM at day 3 and 6 (B) was measured. (A) The total IgM production was comparable between B cells derived from WT and mSIGNR1-deficient mice. (B) Upon stimulation, WT B cells produced more PC-specific IgM compared to B cells derived from mSIGNR1-deficient mice. $^{*}P < 0.05$.

located in the marginal zone of the spleen.

Marginal zone B cells are a unique B cell population, known to be long-lived and to display an activated phenotype [24–26]. Several studies have been performed to investigate the role of cells in the marginal zone of the spleen in T-independent immune responses. It has been described that marginal zone B cells are pivotal for the T-independent immune response against the TI-II antigen Ficoll [27]. Upon elimination of marginal zone macrophages the anti-Ficoll response was diminished [19]. Hence both marginal zone macrophages and marginal zone B cells are crucial for a TI immune response. However, specific depletion of marginal zone macrophages using the anti-SIGNR1 antibody ERTR-9 did not induce a decrease in the TI immune response [20,28].

MARCO, a scavenger receptor that is also expressed by marginal zone macrophages has been described to interact with marginal zone B cells [29]. The interaction between MARCO and an unknown ligand on marginal zone B cells is pivotal for the localization of the marginal zone B cells since disruption of the interaction leads to the migration of marginal zone B cells into the follicle. In addition, Chen *et al.* observed a lower amount of resident peritoneal macrophages and splenic marginal zone macrophages in MARCO-deficient mice [30]. Hence, the interaction of MARCO with juxtaposing marginal zone B cells is important for both marginal zone macrophage and marginal zone B cell localization. However, during ontogeny the absence of MARCO does not affect the localization of marginal zone B cells in the spleen [30].

In this study it was observed that the marginal zone of mSIGNR1-deficient mice contains marginal zone B cells, however their number was decreased in comparison to WT mice. In addition, marginal zone B cells were found to adhere to mSIGNR1 expressed by transfectants, whereas follicular B cells did not interact with mSIGNR1.

The interaction of marginal zone macrophages with marginal zone B cells through mSIGNR1 can have multiple effects. Marginal zone B cells are long-lived and display an activated phenotype [24–26]. In general, activated B cells are prone to go into apoptosis, unless they receive the proper signals for their proliferation. Possibly, marginal zone B cells receive survival signals from neighboring marginal zone macrophages. Since we observed that the marginal zone B cell population is still present in the mSIGNR1-deficient mice, although at a lower level, the absence of mSIGNR1 in these mice could be the cause of a decreased survival signal.

Another explanation for the diminished anti-PC IgM production in the mSIGNR1-deficient mice upon infection with *S. pneumoniae* could be a lack of specificity within the B cell population. Survival signals may also be involved in the composition of the marginal zone B cell repertoire. It has been postulated that marginal zone B cell specificity for antigens such as PC on *S. pneumoniae* arises from the encounter of self-antigens [31,32]. To test the presence of PC-specificity in the mSIGNR1-deficient mice we stimulated splenic B cells and measured both total and PC-specific IgM levels. Strikingly, we observed a decreased level of anti-PC IgM in the mSIGNR1-deficient mice despite a normal value of total IgM.

Based on these results we conclude that mSIGNR1 is involved in the composition of the specificity of the juxtaposed marginal zone B cells. Perhaps mSIGNR1 is involved in the capture and/or presentation of self- and non-self-antigens, resulting in the development of the marginal zone B cell repertoire. However the mechanism behind this phenomenon remains unclear. Recently, Bergtold *et al.* described FcγRIIb-mediated antigen uptake and retention by DC [33]. A proportion of the antigen did not end up in the lysosomal pathway but was retained and conserved for antigen presentation to B cells, facilitating the TI immune response. Possibly antigen uptake by mSIGNR1 may also lead to antigen retention and presentation to the marginal zone B cells, to optimize a swift and accurate early immune response upon infection.

Antigen transfer by marginal zone macrophages to marginal zone B cells has been postulated by van Rooijen *et al.* [13]. Antigen transfer by marginal zone macrophages to marginal zone B cells may optimize the activation status of the marginal zone B cells and may play a role in marginal zone B cell retention in the marginal zone or marginal zone B cell survival, explaining the lower amount of marginal zone B cells in mSIGNR1-deficient mice.

Further research is needed to investigate the cascade of events involved in the early immune response against blood-borne TI-II antigens such as *S. pneumoniae*. Mice deficient for both mSIGNR1 and MARCO and mice deficient for mSIGNR1 but transgenic for PC-specific marginal zone B cells would be powerful tools to analyze the role of

pathogen receptors expressed by marginal zone macrophages in the development of the marginal zone B cell repertoire and marginal zone B cell activation.

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Chapter 8

General discussion of the in vivo function of mSIGNR1

8.1 The in vivo role of mSIGNR1 during infection with *M. tuberculosis*

C-type lectin receptors (CLR) are expressed by antigen presenting cells and recognize specific carbohydrate structures on self-antigens or pathogens. Upon recognition, antigen is internalized, processed and presented [1,2]. In addition, CLR are involved in immune modulation and the induction of tolerance against self-antigens [3]. Hence, specific pathogens target CLR such as DC-SIGN to evade and suppress the immune system [4,5].

The shared binding specificity of DC-SIGN, L-SIGN and mSIGNR1 for mannose-containing structures hinted at the capacity of these molecules to interact with ManLAM, a *Mycobacterium tuberculosis* capsule component with a mannose cap [6]. Indeed all three SIGN molecules specifically interacted with ManLAM and the ara-$(man)_3$ structure was identified as the ligand for DC-SIGN, L-SIGN and mSIGNR1 on *M. tuberculosis* [7]. ManLAM is known as a virulence factor of *M. tuberculosis* and is expressed by slow-growing yet highly pathogenic mycobacteria strains. Previously, ManLAM was identified as an immunomodulating factor responsible for the inhibition of DC maturation and the induction of IL-10 production by DC through DC-SIGN [8]. Therefore, we set out to investigate the in vivo function of mSIGNR1 in an in vivo *M. tuberculosis* infection model (Chapter 4, submitted for publication).

Upon in vitro stimulation of peritoneal macrophages with ManLAM, IL-10 production was induced. In contrast, IL-10 production was abolished in the mSIGNR1-deficient macrophages, indicating that ManLAM binding to mSIGNR1 induces the production of the anti-inflammatory cytokine IL-10. Hence, *M. tuberculosis* is able to induce immunosuppression through mSIGNR1, which may contribute to its survival. Strikingly, IL-10 production upon ligation of DC-SIGN by ManLAM is only induced upon concomitant triggering of TLR4 by LPS [8]. Thus, mSIGNR1 can mediate immune modulation on its own, whereas DC-SIGN-mediated immune modulation through ManLAM is dependent on additional signaling through TLR.

Despite the observed in vitro immune modulation upon ligation of mSIGNR1 with ManLAM, both mice strains succumbed to infection and no differences were observed in the cytokine levels within the lungs. In addition, no expression of mSIGNR1 was induced in the lung upon infection with *M. tuberculosis*. This is in contrast to DC-SIGN, which expression in human lung alveolar macrophages was induced upon infection with *M. tuberculosis* [9]. Therefore, probably due to the lack of mSIGNR1 expression in the lungs, immune modulation does not result in a difference in pathology. Nonetheless, ManLAM may exert modulation of the immune response through mSIGNR1. In the early phase of infection, mSIGNR1 influences the Th1/Th2 balance of the immune response evoked by *M. tuberculosis*. In the mSIGNR1-deficient mice, a Th1 prone response was detected whereas the wild-type mice displayed a mixed Th1/Th2 response. Thus the ManLAM induced IL-10 production by macrophages expressing mSIGNR1 leads to the modulation of the cytokine production by T cells resulting in a mixed Th1/Th2 response. The Th1

prone response observed in the mSIGNR1-deficient mice indicates a Th2-skewing role for mSIGNR1. Perhaps when using a lower dose of *M. tuberculosis*, immune modulation mediated by the interaction of ManLAM and mSIGNR1 will have an effect on the course of the infection.

8.2 mSIGNR1-mediated signaling

DC-SIGN and its homologues are involved in many pivotal functions of the immune system. However, it is still unclear how these C-type lectins are able to exert their function besides the binding to self- and pathogen ligands. It has been shown that DC-SIGN is targeted by pathogens to modulate the immune response and evade their destruction [4]. DC-SIGN is thought to modulate the signaling cascades induced by TLR4 triggering. However, the signaling pathways involved in this mechanism are not clear. Recently, Caparros *et al.* demonstrated using a monoclonal antibody as a ligand for DC-SIGN, that ligation of DC-SIGN induces the phosphorylation of both ERK and Akt [10]. In addition, a transient influx of calcium was induced. These findings fit with the mechanism described, in which DC-SIGN-induced signaling promotes a Th2 prone, more tolerogenic immune response [8,10].

In contrast, Nagoaka *et al.* described that upon concomitant ligation of both TLR4 and mSIGNR1 by LPS from Gram-negative bacteria, mSIGNR1 enhanced the induced cytokine response suggesting a pro-inflammatory role for mSIGNR1 [11]. It remains unclear how mSIGNR1 mediates this response and whether the effect can be attributed solely to mSIGNR1. Possibly the capture of Gram-negative bacteria LPS by mSIGNR1 facilitates other receptors to interact with the pathogen-derived LPS, leading to additional signaling. In addition, the observed pro-inflammatory role of mSIGNR1 by Nagaoka *et al.* is in contrast to the production of the anti-inflammatory cytokine IL-10 observed upon ManLAM stimulation in vitro in Chapter 4.

8.3 The in vivo role of mSIGNR1 during infection with *S. pneumoniae*

The lack of expression of mSIGNR1 by alveolar macrophages probably added to the minor role of mSIGNR1 in the immune response against *M. tuberculosis*. mSIGNR1 is expressed in spleen, liver and lymph node [12]. Since the spleen plays a major role in the immune response against the Gram-negative bacterium *Streptococcus pneumoniae* and pneumococci have been described to target the marginal zone of the spleen, a role for mSIGNR1 in the immune response against *S. pneumoniae* was investigated.

Upon investigation of the binding characteristics of DC-SIGN for a panel of capsular polysaccarides derived from the most common *S. pneumoniae* serotypes causing disease, DC-SIGN displayed specificity for only *S. pneumoniae* serotype 14 polysaccaride. Strik-

ingly, *S. pneumoniae* serotype 14 is a leading cause of invasive pneumococcal disease in the US and Western Europe [13,14]. In addition, DC-SIGN also interacts with encapsulated *S. pneumoniae* serotype 3. The structure on encapsulated serotype 3 that was specifically recognized by DC-SIGN was not detected in the polysaccharide capsule and remains to be identified.

In Chapter 7 we found that the polysaccharide of *S. pneumoniae* serotype 3 does specifically target mSIGNR1 expressing marginal zone macrophages upon intravenous injection in vivo. Hence, mSIGNR1 may bind a specific ligand on the capsule of *S. pneumoniae* serotype 3 that does not bind DC-SIGN, or mSIGNR1 interacts with an epitope that is exposed only in vivo by for example the binding of complement or antibodies.

The specific binding of DC-SIGN to only two of the tested serotypes may add new insights in the pathology of these two pneumococci. The interaction between DC-SIGN and *S. pneumoniae* does not seem to have immunomodulatory consequences. Upon stimulation of DC with *S. pneumoniae* serotype 14 polysaccaride, no modulation of DC biology was observed. Moreover, no immunomodulatory effect to suppress the immune response against pneumococci, similar to the IL-10 production induced by *M. tuberculosis*-derived ManLAM, was detected [8]. Still, the specific binding of *S. pneumoniae* serotype 14 may serve as a tool for the optimization of the vaccines that are currently used. Possibly, the binding specificity of DC-SIGN expressed by immature DC can be used as a tool to target vaccines to the DC. The currently licensed pneumococcal vaccine is based on 23 most common serotypes, and has an overall protective efficacy of about 60-70% [15]. The targeting of such a vaccine to the DC via the specific binding of serotype 14 polysaccharides to DC-SIGN may enhance the immune response against all 23 pneumococcal serotypes. Future research should look into the use of such an approach.

The spleen plays an important role in the immune defense against *S. pneumoniae* [16]. In addition, *S. pneumoniae* and its polysaccharides have been described to target the marginal zone of the spleen. Possibly, mSIGNR1 is involved in the specific uptake and clearance of blood-borne *S. pneumoniae* by the spleen, resulting in effective immunity. Indeed, mSIGNR1 specifically interacts with both *S. pneumoniae* serotype 3 and 14 [17,18]. Moreover, Lanoue *et al.* describe that mSIGNR1 is beneficial for the immune defense against pneumococci [18]. The intraperitoneal infection model used by Lanoue *et al.* demonstrated the pivotal role for mSIGNR1 as a pathogen receptor expressed by peritoneal macrophages, facilitating the clearance of the bacteria in the peritoneal cavity.

In Chapter 6 the role of mSIGNR1 during infection with *S. pneumoniae* serotype 3 was investigated using an infection model resembling the natural route of entry [19]. Similar to the intraperitoneal infection model, mSIGNR1-deficient mice succumb to infection with *S. pneumoniae* in contrast to wild-type mice. Investigation of the sera of the mice during infection demonstrated that in wild-type mice antibodies against the *S. pneumoniae* capsular component phosphorlycholine (PC) were raised, in contrast to the levels of early anti-PC antibodies in mSIGNR1-deficient mice.

Marginal zone B cells together with peritoneal B-1 cells are involved in the produc-

tion of these early antibodies that are protective upon *S. pneumoniae* infection [20,21]. Upon investigation of the capture of blood-borne *S. pneumoniae*, we observed that *S. pneumoniae* was not captured by marginal zone macrophages of SIGNR1-deficient mice. Thus, mSIGNR1 expressed by marginal zone macrophages captures blood-borne *S. pneumoniae* and can be involved in the production of early antibodies by marginal zone B cells. Marginal zone B cells reside in close proximity to marginal zone macrophages in the marginal zone of the spleen [22]. mSIGNR1 may mediate antigen transfer to the marginal zone B cell population or directly activate marginal zone B cells to produce these early antibodies. Indeed, marginal zone macrophages are important for the function of marginal zone B cells since Karlsson *et al.* described that the interaction between MARCO expressed by marginal zone macrophages and marginal zone B cells is pivotal for marginal zone B cell localization in the marginal zone [23]. Strikingly, mSIGNR1-deficient mice display a reduction in the marginal zone B cell population, suggesting that mSIGNR1 is involved in either retention or survival of marginal zone B cells in the marginal zone. Moreover, an in vitro cell-cell adhesion assay demonstrated that mSIGNR1 specifically interacts with marginal zone B cells in contrast to follicular B cells, suggesting a direct interaction between marginal zone macrophages and marginal zone B cells through mSIGNR1. Furthermore, the response to LPS by mSIGNR1-deficient and wild-type mice-derived splenic B cells resulted in a similar production of IgM. However, the mSIGNR1-deficient mice-derived splenic B cells produced a reduced amount of anti-PC IgM upon polyclonal stimulation. These findings indicate that mSIGNR1 expressed by marginal zone macrophages is involved in the composition of the marginal zone B cell population and repertoire.

8.4 The role of mSIGNR1 in the early immune response against blood-borne pathogens

Marginal zone B cells form a B cell population distinct from peritoneal B-1 and follicular recirculating B-2 cells. This compartmentalization may be crucial for the specialized function of marginal zone B cells; the execution of the early immune response against blood-borne antigens. The marginal zone B cell repertoire is not a random repertoire; certain B cell receptors are enriched in this population [24]. The mechanism behind this enrichment of certain clones has not been elucidated completely but there is a role for B cell receptor associated Brutons tyrosine kinase and coreceptor CD19. In addition, these B cell receptors display a specificity for autoantigens or commensal flora [24–26]. Possibly, (auto)antigens play a role in the enrichment and recruitment of certain specificities into the marginal zone population.

It has been described that *S. pneumoniae* displays molecular mimicry with the autoantigen oxidized low-density lipoprotein and some antibodies against oxidized low-density lipoprotein recognize *S. pneumoniae* phosphorylcholine [27]. In addition, DC-SIGN

specifically binds to oxidized low-density lipoprotein in contrast to the non-oxidized form [28]. Hence, mSIGNR1 may also be involved in the capture of oxidized low-density lipoprotein resulting in the manifestation of anti-phosphorylcholine specificity in the marginal zone B cell repertoire. Thus, mSIGNR1 may be involved in the capture of (auto)antigens pivotal for the recruitment and stimulation of marginal zone B cells reactive to these antigens. This mechanism provides a marginal zone B cell repertoire that despite the low affinity for certain autoantigens is equipped to respond fast to blood-borne pathogens by recognition of common structures expressed by these pathogens. However, upon distortion of this balance, autoimmunity may ensue. Thus, mSIGNR1 could be involved in the development of the marginal zone B cell repertoire resulting in a reduced population with less specificities in the mSIGNR1-deficient mouse strain and may be involved in the prevention of autoimmunity. More research is needed to elucidate the functional consequences of the interaction of mSIGNR1 expressed by marginal zone macrophages and marginal zone B cells on both the marginal zone B cell population and repertoire. Identification of the ligand expressed on the marginal zone B cells may provide more insight in this interaction.

8.5 Concluding remarks

In this thesis the in vivo function of mSIGNR1 was investigated. Besides its similarities to both DC-SIGN and L-SIGN, leading to its function as a model to study the in vivo role of these two C-type lectins, mSIGNR1 displays a unique function as a pathogen receptor pivotal for the early immune response against blood-borne antigens. Recently Kang *et al.* described that mSIGNR1 binds the complement component C1q which results in the fixation of C3. Possibly, this mechanism contributes to the clearance of *S. pneumoniae* and may be involved in the activation of marginal zone B cells [29].

In contrast, DC-SIGN has been described to be targeted by pathogens to modulate and concomitantly evade the immune response [4]. The fact that mSIGNR1 plays a crucial role in the early immune response against *S. pneumoniae* poses the question whether DC-SIGN is also involved in the immune defense against pathogens. Possibly, immune modulation by pathogens through DC-SIGN should be regarded as an exception. Thus far, research has focused on the interaction of DC-SIGN with T cells, endothelial cells, and neutrophils [30–35]. The interaction between mSIGNR1 and marginal zone B cells suggests that also DC-SIGN may interact with specific B cells populations. Thus, DC-SIGN might also have a functional role in autoimmunity and T-independent immune responses.

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Summary

Several studies have been performed to investigate the function of both human DC-SIGN and L-SIGN. However, thus far, research was limited to in vitro and ex vivo studies. To understand the potential functions of these molecules in vivo, we set out to investigate the function of the murine homologue SIGNR1.

The carbohydrate binding characteristics of mSIGNR1 were compared to DC-SIGN and L-SIGN in Chapter 2[1]. For the large part, DC-SIGN, L-SIGN and mSIGNR1 display similar binding characteristics such as the binding to high mannose structures. It has been described that viruses such as HIV-1, lymphocytic choriomeningitis virus (LCMV) and the severe acute respiratory syndrome (SARS) coronavirus bind DC-SIGN through high mannose structures. Thus, it is likely that mSIGNR1 also binds these viruses. Consequently, mSIGNR1 can be used as a model for both DC-SIGN and L-SIGN to study the functional consequences of the binding of high mannose structures expressed by both host and pathogen. DC-SIGN and L-SIGN display a difference in the ability to bind to Lewis^x. mSIGNR1 has the capacity to bind Lewis^x, similar to DC-SIGN and in contrast to L-SIGN[2,3]. Hence, we concluded that the binding capacity of mSIGNR1 is more similar to DC-SIGN than to L-SIGN and that pathogens such as the Gram-negative *H. pylori* and the worm parasite *Schistosoma mansoni* may also interact with mSIGNR1 through the expression of Lewis antigens. Indeed, mSIGNR1 interacts with *H. pylori* LPS rich in Lewis^x and Lewis^y[1].

We also demonstrated a unique binding specificity of mSIGNR1 for sialated-Lewis^x in contrast to both DC-SIGN and L-SIGN. Sialated-Lewis^x is an L-, E- and P-selectin ligand and mSIGNR1 may share their affinity for sialylated Lewis antigens. Therefore, the interaction with sialyl Lewis^x may facilitate the binding of sialyl Lewis^x-expressing lymphocytes with mSIGNR1.

mSIGNR1, similar to DC-SIGN and L-SIGN, binds the capsular component of *Mycobacterium tuberculosis* ManLAM (Chapter 3)[4]. The interaction of ManLAM with DC-SIGN expressed by immature DC results in the arrest of DC maturation and enhanced IL-10 production[5]. ManLAM, which is also shed by the mycobacterium into the blood, uses the binding to DC-SIGN to modulate the immune response for its own benefit[5]. We

identified the structure expressed by ManLAM that is recognized by DC-SIGN, L-SIGN and mSIGNR1. Using synthesized structures, we demonstrated that the SIGN molecules specifically target the mannosylated cap of ManLAM, with the highest affinity for the $(man)_3$-ara structure compared to $(man)_2$-ara and man-ara structures.

Since mSIGNR1 specifically binds ManLAM and *M. tuberculosis*, the in vivo function of mSIGNR1 during infection with *M. tuberculosis* was investigated (Chapter 4, submitted for publication). Although mSIGNR1 is not expressed by alveolar macrophages [6], systemically secreted ManLAM may exert immune modulation through mSIGNR1. In addition, expression of DC-SIGN was described to be induced upon infection with *M. tuberculosis* [7]. Possibly, mSIGNR1 expression by alveolar macrophages is also induced upon infection, facilitating a scavenging role for mSIGNR1 in the lung during infection.

In vitro stimulation of peritoneal macrophages of both wild-type and mSIGNR1-deficient mice demonstrated that mSIGNR1 induced an increase in IL-10 production by these cells. These in vitro results suggested that mSIGNR1, similar to DC-SIGN, can be used by *M. tuberculosis* to modulate the immune system, to enhance its propagation and pathology. However, upon infection with *M. tuberculosis*, both mice strains succumbed to this pathogen and no differences in local IL-10 or other cytokine production was apparent during infection. More T cell activation in mSIGNR1-deficient mice was observed early during infection. In addition, the mSIGNR1-deficient mice displayed an increase in the Th1 associated cytokine IFNγ and a trend towards a lower production of the Th2 associated cytokine IL-4 upon restimulation of splenocytes with PPD. Hence, mSIGNR1 expressed by macrophages may be involved in the production of cytokines by T cells. However, no difference in T cell recruitment was observed. Thus, *M. tuberculosis* is capable to bind mSIGNR1, and this C-type lectin may be involved in the orchestration of the Th1/Th2 immune balance. However, mSIGNR1 does not play a significant role in *M. tuberculosis* pathology, probably due to the expression pattern of mSIGNR1 at remote sites such as the splenic marginal zone but not by alveolar macrophages.

In contrast to *M. tuberculosis*, the spleen plays an important role in the defense against *Streptococcus pneumoniae* [8]. First the interaction of DC-SIGN with a panel of *S. pneumoniae* polysaccharides of various serotypes was investigated (Chapter 5) [9]. DC-SIGN selectively binds *S. pneumoniae* serotype 3 and 14, and the polysaccharide of *S. pneumoniae* serotype 14. In vitro experiments showed that serotype 14 polysaccharides do not modulate DC biology and do not suppress the immune response against pneumococci as is the case for *M. tuberculosis*-derived ManLAM [5]. Thus the implications of the binding of *S. pneumoniae* to DC-SIGN are currently unknown. In vivo experiments have shown that *S. pneumoniae* polysaccharides specifically target to the marginal zone of the spleen [10,11]. In addition, the spleen is pivotal for an appropriate immune response against this pathogen [12]. Therefore, the expression of mSIGNR1 by marginal zone macrophages hints to a role of this C-type lectin in the immune response against *S. pneumoniae*. Indeed, similar to DC-SIGN, mSIGNR1 also specifically interacts with both *S. pneumoniae* serotype 3 and 14 (Chapter 6) [11,13,14]. The role of mSIGNR1 during infection with *S. pneumo-*

niae serotype 3 was investigated using an mSIGNR1-deficient mouse strain (Chapter 6) [14]. Infection was induced by the administration of the serotype 3 pneumococci in the upper airways, resembling the natural route of entry. Strikingly, mSIGNR1-deficient mice succumb to infection with *S. pneumoniae*, in contrast to wild-type mice. Upon investigation of the sera of the mice during pneumococcal infection, the wild-type levels of early antibodies against the pneumococcal antigen phosphorylcholine (PC) were raised during infection, in contrast to the levels of early anti-PC IgM in mSIGNR1-deficient mice, which remained low. Marginal zone B cells together with peritoneal B-1 cells are involved in the production of these early antibodies. Marginal zone B cells reside in close proximity to marginal zone macrophages in the marginal zone of the spleen. Therefore, we hypothesized that mSIGNR1, through the capture of *S. pneumoniae*, is involved in the production of early antibodies by marginal zone B cells. mSIGNR1 may mediate antigen transfer to the marginal zone B cell population or directly activate marginal zone B cells to produce these early antibodies. This subject was further explored in Chapter 7, and it was found that marginal zone B cells specifically bind mSIGNR1, in contrast to follicular B cells. In addition, mSIGNR1-deficient mice displayed a reduction in their marginal zone B cell population, suggesting that mSIGNR1 is involved in the retention or survival of marginal zone B cells in the marginal zone. Upon polyclonal stimulation, mSIGNR1-deficient mice-derived splenic B cells produced a reduced amount of anti-PC IgM whereas the total amount of IgM was similar to their wild-type counterparts.

The reduced marginal zone B cell population, with a lower amount of PC-specific cells observed in the mSIGNR1-deficient mice, indicates that mSIGNR1 is involved in the development of the marginal zone B cell repertoire. However, the underlying mechanism remains to be elucidated. Possibly mSIGNR1 expressed by marginal zone macrophages provides survival signals or mediates retention of the marginal zone B cells.

In conclusion, mSIGNR1 is involved in the early immune defense against *S. pneumoniae* infection. Thus far, DC-SIGN was demonstrated to be hijacked by pathogens in order to evade an immune response but our data suggest that members of the SIGN family can also be involved in the immune defense against pathogens. In addition, we describe that mSIGNR1 is involved in the immune defense against pneumococcal infection through the interaction with marginal zone B cells. DC-SIGN expressed by DC has been described to interact with T cells in both the immunological and infectious synapse where viruses are transmitted [15,16]. Future studies should look into the interaction between mSIGNR1 and DC-SIGN with B cells and the concomitant functional consequences such as antigen presentation to B cells and B cell activation.

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Samenvatting in het Nederlands voor niet-immunologen

Het menselijk lichaam is in staat om zichzelf te beschermen tegen een groot aantal ziekteverwekkers, zoals bacteriën, schimmels, parasieten en virussen; dit worden ook wel pathogenen genoemd. De cellen en processen die hierbij betrokken zijn vormen samen het afweersysteem, ook wel immuunsysteem genaamd. Een belangrijk proces binnen het immuunsysteem is het herkennen van ziekteverwekkers in een vroeg stadium van een eventuele infectie.

Overal in ons lichaam en vooral op plaatsen waar ziekteverwekkers het lichaam binnen kunnen komen, zoals de mond, de neus, de longen, bij de darmen, maar ook in de huid, liggen cellen van het immuunsysteem te wachten op ziekteverwekkers. Deze cellen, zogezegd de "poortwachters" van het immuunsysteem, zijn in staat een groot aantal ziekteverwekkers te herkennen en te binden. De herkenning van pathogenen is mogelijk doordat deze cellen specifieke eiwitten op het celmembraan hebben, de receptoren, die structuren die veel voorkomen bij ziekteverwekkers kunnen herkennen. Deze structuren, die kunnen worden gebonden door receptoren, worden liganden genoemd.

De poortwachtercellen van het immuunsysteem zijn niet allemaal hetzelfde. Een van de soorten poortwachtercellen zijn de dendritische cellen. Deze cellen hebben lange uitlopers (dendron is Grieks voor boom) waarmee ze continu de omgeving aftasten om ziekteverwekkers op te sporen. Wanneer ze een ziekteverwekker herkennen en binden, verplaatsen ze zich naar de lymfeklieren om de afweerreactie op gang te brengen door witte bloedcellen, de T-cellen, te activeren.

Een van de receptoren waarmee de dendritische cel een ziekteverwekker kan herkennen heet DC-SIGN. Dit is een receptor die alleen voorkomt op de dendritische cel. Naast het herkennen van ziekteverwekkers heeft deze receptor nog andere functies. Wanneer dendritische cellen net aangemaakt zijn en zich in het bloed bevinden speelt DC-SIGN een belangrijke rol bij de migratie uit het bloed richting weefsels zoals de huid. Zoals al eerder is beschreven, vertrekken de dendritische cellen zodra ze een ziekteverwekker hebben herkend naar de lymfeklieren om hier T-cellen te activeren. DC-SIGN is ook

betrokken bij het contact maken met deze T-cellen.

Echter, DC-SIGN is vooral bekend vanwege het feit dat het specifiek kan binden aan het Humane Immunodeficiëntie Virus (HIV). Dendritische cellen met DC-SIGN op het oppervlak bevinden zich bij de slijmvliezen van de geslachtsorganen, de mond en de anus; plaatsen waar HIV door seksueel contact kan worden overgedragen van mens tot mens. Wanneer HIV door de slijmvliezen heen het lichaam binnen probeert te komen kan de dendritische cel via DC-SIGN HIV binden. Vervolgens migreert de dendritische cel naar de lymfeknoop om T-cellen te activeren. Echter, de binding van DC-SIGN aan HIV leidt niet tot een verbeterde immuunrespons en het voorkomen van HIV-infectie: HIV profiteert juist van de binding aan DC-SIGN op de dendritische cel. HIV verstopt zich in de dendritische cel en laat zich meevoeren richting de T-cellen in de lymfeklieren. De T-cellen zijn namelijk de "slachtoffercellen" van HIV. Door specifiek deze T-cellen aan te vallen zorgt HIV ervoor dat het immuunsysteem van de patiënt stil komt te liggen met de ziekteverschijnselen van AIDS tot gevolg. Onze groep heeft aangetoond dat DC-SIGN het niet alleen mogelijk maakt dat HIV mee kan liften naar de T-cellen maar dat DC-SIGN ook nog helpt bij het infecteren van de T-cel met HIV. Wanneer DC-SIGN aanwezig is kan HIV meer T-cellen infecteren dan bij de afwezigheid van DC-SIGN.

Sinds de ontdekking van DC-SIGN door onze groep is er al veel onderzoek gedaan naar de functie van DC-SIGN. Ook wordt er door onze groep onderzoek gedaan naar een homoloog van DC-SIGN in de mens; L-SIGN. Deze homoloog komt voor op bepaalde cellen in de lever en de lymfeklier. Echter, het onderzoek bestaat voornamelijk uit experimenten die met losse cellen in kweekflesjes (in vitro) worden uitgevoerd. Experimenten met mensen zijn wel mogelijk maar alleen als er al heel veel voorwerk is verricht met experimenten in dieren. Een voor de hand liggend dier om experimenten mee te doen in de immunologie is de muis. Er zijn namelijk heel veel ziektes die ook kunnen worden opgewekt in de muis en vervolgens worden bestudeerd: de infectiemodellen. Ook is er veel kennis op het gebied van het maken van muizen die een bepaald gen missen, de knock-outmuizen.

Het doel van dit promotieonderzoek was het onderzoeken van DC-SIGN in de muis. Er bleken wel 5 DC-SIGN-achtige moleculen (DC-SIGN homologen) te zijn in de muis (mDC-SIGN, mSIGNR1, mSIGNR2, mSIGNR3 en mSIGNR4) waarvan mDC-SIGN en mSIGNR1 tot expressie komen op immunologisch interessante cellen; respectievelijk de dendritische cel en de macrofaag. Hoewel mDC-SIGN een veelbelovende kandidaat voor onderzoek aan DC-SIGN was doordat het net als DC-SIGN specifiek tot expressie komt op de dendritische cel, bleek dat deze receptor de liganden die DC-SIGN kon binden allemaal niet bond. Een andere homoloog, mSIGNR1, kon wel binden aan dezelfde liganden als DC-SIGN. Vandaar dat dit promotieonderzoek zich heeft gericht op de functie van mSIGNR1, een homoloog van DC-SIGN in de levende muis (in vivo).

Zoals al eerder genoemd, komt mSIGNR1 niet voor op het oppervlak van de dendritische cel, maar op een specifieke groep macrofagen in de milt. Macrofagen (vrij vertaald "veelvraten") zijn immuuncellen die net als dendritische cellen door het hele lichaam

verspreid liggen, en dode of beschadigde cellen, maar ook pathogenen kunnen opnemen en verteren.

Om zo snel mogelijk een effectieve immuunreactie te genereren zijn er op diverse plaatsen in ons lichaam verschillende organen van het immuunsysteem gesitueerd. Een aantal van die organen zijn de honderden lymfeklieren die zich op strategische plekken in het lichaam bevinden. De milt is een ander orgaan van het immuunsysteem en is veel groter dan een lymfeklier. Het is een vuistgroot, zeer goed doorbloed orgaan dat ligt onder het middenrif, achter de maag. Zoals lymfeklieren de huid controleren op indringers, zo controleert de milt het bloed op pathogenen. Naast deze immunologische functie is de milt ook belangrijk voor het afbreken van verouderde rode bloedcellen en het recyclen van het ijzer dat vrijkomt bij deze afbraak. Aangezien de meeste taken van de milt kunnen worden overgenomen door andere organen, is het mogelijk dat individuen bij wie de milt operatief is verwijderd een relatief normaal leven kunnen leiden. Echter, de afweer tegen bacteriën die zijn omgeven door een kapsel met suikerstaarten, zoals *Streptococcus pneumoniae*, is verminderd wanneer de milt is verwijderd. *Streptococcus pneumoniae* is een ziekteverwekker die verantwoordelijk is voor onder andere hersenvliesonststeking (nekkramp), longontsteking en oorinfecties. Vandaar dat patiënten, bij wie de milt zal worden verwijderd, gevaccineerd worden.

De structuur van het miltweefsel laat witte bolvormige structuren zien binnen een rode sponzige massa. De witte structuur wordt de witte pulpa genoemd. Deze structuur bevat de witte bloedcellen; de T- en B-cellen, die verantwoordelijk zijn voor de immunologische functie van de milt. De rode massa is rood vanwege de rijke doorbloeding en wordt de rode pulpa genoemd. In de rode pulpa worden de verouderde rode bloedcellen uit het bloed gefilterd en afgebroken. De rode en witte pulpa worden van elkaar gescheiden door de marginale zone. Deze marginale zone bestaat uit een bloedvat waar een deel van het arteriële bloed in terecht komt maar waar de snelheid van de bloedsomloop drastisch is verlaagd, en diverse immuuncelpopulaties. De marginale zone macrofagen liggen tussen het bloedvat en de rode pulpa. Door specifieke technieken waarbij specifieke eiwitten op het celmembraan van cellen zichtbaar gemaakt kunnen worden, de immunohistochemie, kunnen we diverse populaties macrofagen van elkaar onderscheiden.

De marginale zone macrofaagpopulatie werd al in de jaren ’80 ontdekt doordat zij specifiek reageerde met de antistof ERTR-9. Heel lang was het onbekend wat ERTR-9 precies herkende op het celmembraan van de marginale zone macrofaag. Vlak voor de start van dit promotieonderzoek heeft onze groep ontdekt dat ERTR-9 specifiek mSIGNR1 (een muis-homoloog van DC-SIGN) herkent. Het doel van het promotieonderzoek was het vergroten van het inzicht in de functie van mSIGNR1. Door de eigenschappen van mSIGNR1 te onderzoeken en te vergelijken met DC-SIGN en L-SIGN, kunnen we erachter komen in hoeverre mSIGNR1 in de muis te vergelijken is met DC-SIGN in de mens. Vervolgens kunnen muismodellen, waarbij de functie van mSIGNR1 wordt onderzocht in het levende dier, ons meer informatie geven over de functie van DC-SIGN en L-SIGN in de mens.

In hoofdstuk 1 wordt een overzicht gegeven van de literatuur op het gebied van DC-SIGN en de homologen van DC-SIGN in mens en muis. Aangezien mSIGNR1 op een specifieke macrofaagpopulatie in de milt tot expressie komt, wordt in dit hoofdstuk de anatomie van de milt uitgebreid besproken.

In hoofdstuk 2 worden de bindingseigenschappen van mSIGNR1 bestudeerd. Aangezien het hoofddoel van het promotieonderzoek is om door in vivo experimenten met een muis-homoloog van DC-SIGN meer kennis te verkrijgen over de in vivo functie van DC-SIGN worden de bindingseigenschappen vergeleken met DC-SIGN. Ook L-SIGN wordt in deze studie betrokken aangezien studies aan mSIGNR1 wellicht ook meer informatie zouden kunnen geven over L-SIGN. De binding van diverse suikerstructuren aan mSIGNR1, DC-SIGN en L-SIGN wordt vergeleken. De drie homologen vertonen in grote lijnen hetzelfde bindingspatroon. Ze binden bijvoorbeeld alledrie stucturen met een groot aantal mannosegroepen. Door deze kennis weten we dat mSIGNR1 gebruikt kan worden als model voor zowel DC-SIGN als L-SIGN bij het bestuderen van de in vivo functie van deze moleculen, bij het binden aan deze structuren op lichaamseigen structuren of op pathogenen. Naast overeenkomsten zijn er echter ook verschillen. DC-SIGN bindt aan de suikerstructuur "Lewis^x" terwijl L-SIGN deze structuur niet bindt. mSIGNR1 kan net zoals DC-SIGN wel binden aan Lewis^x. Dus kan mSIGNR1 model staan voor DC-SIGN bij het bestuderen van de interactie met Lewis^x. Echter, qua expressie lijkt mSIGNR1 meer op L-SIGN dan op DC-SIGN aangezien mSIGNR1 net als L-SIGN tot expressie komt op specifieke cellen in de lever en de lymfeklier, en niet op dendritische cellen zoals DC-SIGN. Ook blijkt uit deze bindingsstudie dat mSIGNR1 kan binden aan Lewis^x met een siaalzuurgroep terwijl zowel DC-SIGN als L-SIGN dit niet doen. Dit suggereert dat mSIGNR1 ook functies zou kunnen hebben die niet vergelijkbaar zijn met DC-SIGN of L-SIGN.

Nu we meer weten over de bindingseigenschappen van mSIGNR1 kunnen we proberen te voorspellen welke ziekteverwekkers deze receptor zal kunnen binden. Het binden aan Lewis-antigenen wijst er bijvoorbeeld op dat mSIGNR1 zal kunnen binden aan de parasitaire worm *Schistosoma mansoni* of de bacterie *Helicobacter pylori* (die maagzweren kan veroorzaken), aangezien het bekend is dat deze ziekteverwekkers Lewis-structuren op hun oppervlak hebben.

Ook zijn DC-SIGN, L-SIGN en mSIGNR1 alledrie in staat om ManLAM, een onderdeel van het kapsel van *Mycobacterium tuberculosis* te binden. *Mycobacterium tuberculosis* veroorzaakt tuberculose bij de mens. Onze groep heeft al eerder laten zien dat ManLAM-binding aan DC-SIGN op humane dendritische cellen in vitro ertoe leidt dat dendritische cellen uitgeschakeld worden en een stofje (interleukine-10) gaan produceren dat de immuunrespons afremt. De kapselcomponent ManLAM wordt expres door de mycobacterie uitgescheiden om via DC-SIGN de afweerreactie te onderdrukken, waardoor *Mycobacterium tuberculosis* beter kan gedijen.

In hoofdstuk 3 hebben we uitgezocht welke structuur op ManLAM nu precies gebonden wordt door DC-SIGN. Het zijn de mannosegroepen van ManLAM. ManLAM bestaat

uit een polymeer van arabinose waar op drie plekken verschillende hoeveelheden mannosegroepen gebonden kunnen zijn. De binding aan DC-SIGN, L-SIGN en mSIGNR1 is het beste als er meer dan 1 mannosegroep per plek aanwezig is. Nu we weten welke structuren verantwoordelijk zijn voor de binding van ManLAM aan DC-SIGN is het mogelijk om middelen te verzinnen om deze interactie te blokkeren zodat *Mycobacterium tuberculosis* niet langer in staat is het immuunsysteem te onderdrukken. Echter het onderdrukken van het immuunsysteem door ManLAM-binding aan DC-SIGN is tot dusver alleen in vitro aangetoond.

Om de rol van DC-SIGN tijdens een infectie met *Mycobacterium tuberculosis* verder te onderzoeken hebben we in hoofdstuk 4 een *Mycobacterium tuberculosis* infectiemodel bestudeerd met mSIGNR1 knock-outmuizen. In vitro stimulatie van macrofagen met en zonder mSIGNR1 laten zien dat mSIGNR1 net als DC-SIGN ook interleukine-10 aanmaakt na stimulatie. Dit is een aanwijzing dat ook mSIGNR1 door *Mycobacterium tuberculosis* gebruikt kan worden om de afweerreactie te remmen. Echter, na infectie van normale (wild-type-) en knock-outmuizen met *Mycobacterium tuberculosis* waren er geen verschillen in het ziektebeeld van de muizen te meten. Wel hebben we een aantal aanwijzingen dat mSIGNR1 een rol speelt in de "aard" van de immuunreactie. Een verklaring voor het ontbreken van grote verschillen in ziektebeeld tussen de wild-type- en knock-outmuizen is dat mSIGNR1 tot expressie komt in de milt, de lever en in de buikholte, terwijl *Mycobacterium tuberculosis* vooral actief is in de long. Hierdoor kan een eventuele invloed van mSIGNR1 uiteindelijk geen meetbare invloed hebben op het ziektebeeld van de muizen.

Het is bekend dat de milt een belangrijke rol speelt bij de afweer tegen de bacterie *Streptococcus pneumoniae*. In hoofdstuk 5 laten we zien dat DC-SIGN in staat is om deze bacterie te binden maar deze binding leidt, in tegenstelling tot de binding van ManLAM op *Mycobacterium tuberculosis*, niet tot onderdrukking van het immuunsysteem. In hoofdstuk 6 bestuderen we een infectiemodel met *Streptococcus pneumoniae*. De muizen worden geïnfecteerd door ze kleine druppeltjes met bacterie op te laten snuiven, wat vergelijkbaar is met een besmetting die je oploopt doordat iemand anders hoest. Nu waren er grote verschillen tussen de wild-type- en knock-outmuizen waar te nemen; de knock-outmuizen waren veel zieker dan de wild-typemuizen. Toen we het bloed van de muizen nader gingen onderzoeken bleek dat de wild-typemuizen tijdens de infectie een bepaalde antistof hadden aangemaakt; anti-fosforylcholine, terwijl deze antistof amper aanwezig was in de knock-outmuizen. Het is bekend dat deze anti-fosforylcholine antistof de *Streptococcus pneumoniae* specifiek kan herkennen en zo een onderdeel vormt van de afweerreactie tegen deze ziekteverwekker. De antistof wordt gemaakt door specifieke B-cellen in de buikholte en de marginale zone van de milt. Deze B-cellen zijn bijzonder omdat ze zonder hulp van T-cellen antistoffen kunnen produceren. Het voordeel hiervan is dat er binnen zeer korte tijd een antistof geproduceerd kan worden waardoor de ziekteverwekker snel onschadelijk kan worden gemaakt.

Marginale zone B-cellen bevinden zich vlakbij de marginale zone macrofagen met

mSIGNR1 op het oppervlak. Wellicht dat mSIGNR1 door het binden en wegvangen van *Streptococcus pneumoniae* betrokken is bij de produktie van de "vroege" antistoffen door marginale zone B-cellen. In hoofdstuk 7 hebben we een begin gemaakt met het onderzoek naar de rol van mSIGNR1 bij de productie van antistoffen door marginale zone B-cellen. Ons onderzoek wijst uit dat mSIGNR1 kan binden aan marginale zone B-cellen. Bovendien waren er veel minder marginale zone B-cellen aanwezig in de milt van mSIGNR1 knock-outmuizen dan in de milt van wild-typemuizen. Deze resultaten wijzen er op dat mSIGNR1 betrokken kan zijn bij het "aantrekken" van B-cellen naar de marginale zone: dat in de afwezigheid van mSIGNR1 de marginale zone B-cellen de marginale zone niet kunnen vinden en hier dus niet terecht komen. Ook is het mogelijk dat marginale zone B-cellen afhankelijk zijn van een "overlevingssignaal" van mSIGNR1 waardoor ze in de knock-outmuis sneller doodgaan.

Wanneer B-cellen uit de milt van knock-out- of wild-typemuizen in vitro gestimuleerd werden om antistof te produceren was er wederom een verschil te meten in de hoeveelheid anti-fosforylcholine antistof. De B-cellen uit de milt van de knock-outmuis produceerden wel wat anti-fosforylcholine antistof maar dit was veel minder dan de hoeveelheid geproduceerd door de B-cellen uit de milt van de wild-typemuis. Wellicht is mSIGNR1 betrokken bij het samenstellen van het repertoire van de B-cellen door fosforylcholine-specifieke B-cellen aan te trekken en/of in leven te houden.

Samenvattend beschrijft dit proefschrift de complexe rol van mSIGNR1 in vitro en in vivo. mSIGNR1 is een homoloog van DC-SIGN en kan model staan voor deze receptor. Echter doordat mSIGNR1 niet zoals DC-SIGN tot expressie komt op de dendritische cel maar op specifieke macrofaagpopulaties in de milt, lymfeklier, buikholte en op endotheelcellen van de lever is mSIGNR1 geen ideale, maar tot dusver wel de enige kandidaat voor het verkrijgen van informatie over de in vivo functie van DC-SIGN. Juist vanwege dit expressiepatroon is mSIGNR1 een zeer geschikte kandidaat voor het onderzoeken van de in vivo functie van L-SIGN. Er is een stuk minder bekend over de functie van L-SIGN dan die van DC-SIGN; waarschijnlijk zal verder onderzoek naar mSIGNR1 ook nieuwe invalshoeken genereren voor het onderzoek naar L-SIGN en de immunologie in het algemeen.

Tijdens dit promotieonderzoek is duidelijk geworden dat mSIGNR1, naast de overeenkomsten met DC-SIGN en L-SIGN, een unieke functie heeft als pathogeenreceptor en betrokken is bij de snelle afweerreactie tegen ziekteverwekkers in het bloed.

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Stella

List of publications

- C.W. Wieland, E.A. Koppel, J. den Dunnen, S. Florquin, A.N.J. McKenzie, Y. van Kooyk, T. van der Poll and T.B.H. Geijtenbeek. Mice lacking murine SIGNR1 have a normal host defense against *Mycobacterium tuberculosis*. *Submitted.* (Chapter 4)
- E.A. Koppel, C.W. Wieland, V.C.M. van den Berg, M. Litjens, S. Florquin, Y. van Kooyk, T. van der Poll and T.B.H. Geijtenbeek. Specific ICAM-3 grabbing nonintegrin-related 1 (SIGNR1) expressed by marginal zone macrophages is essential for defense against pulmonary *Streptococcus pneumoniae* infection. *Eur. J. Immunol.* 2005; 35 (10): 2962-2969. (Chapter 6)
- E.A. Koppel, E. Sæland, D.J.M. de Cooker, Y. van Kooyk and T.B.H. Geijtenbeek. DC-SIGN specifically recognizes *Streptococcus pneumoniae* serotypes 3 and 14. *Immunobiology* 2005; 210 (2-4): 203-210. (Chapter 5)
- E.A. Koppel, I.S. Ludwig, B.J. Appelmelk, Y. van Kooyk and T.B.H. Geijtenbeek. Carbohydrate specificities of the murine DC-SIGN homologue mSIGNR1. *Immunobiology* 2005; 210 (2-4): 195-201. (Chapter 2)
- E.A. Koppel, K.P.J.M. van Gisbergen, T.B.H. Geijtenbeek and Y. van Kooyk. Distinct functions of DC-SIGN and its homologues L-SIGN (DC-SIGNR) and mSIGNR1 in pathogen recognition and immune regulation. *Cellular Microbiology* 2005; 7 (2): 157-165. (Chapter 1)
- E.A. Koppel, I.S. Ludwig, M. Sanchez Hernandez, T.L. Lowary, R.R. Gadikota, A.B. Tuzikov, C.M.J.E. Vandenbroucke-Grauls, Y. van Kooyk, B.J. Appelmelk and T.B.H. Geijtenbeek. Identification of the mycobacterial carbohydrate structure that binds the C-type lectins DC-SIGN, L-SIGN and SIGNR1. *Immunobiology* 2004; 209 (1-2): 117-127. (Chapter 3)
- T.B.H. Geijtenbeek, S.J. van Vliet, E.A. Koppel, M. Sanchez Hernandez, C.M.J.E. Vandenbroucke-Grauls, B.J. Appelmelk and Y. van Kooyk. Mycobacteria target DC-SIGN to suppress dendritic cell function. *J. Exp. Med.* 2003; 197 (1): 7-17.

Curriculum Vitae

Estella Adriana Koppel werd geboren op 14 augustus 1978 te Rotterdam. In 1996 behaalde zij haar VWO-diploma aan het Goois Lyceum te Bussum. In hetzelfde jaar begon zij de studie Medische Biologie aan de Universiteit Utrecht. De eerste wetenschappelijke stage bracht zij door op het laboratorium Immunotherapie onder leiding van prof.dr. J.G.J. van de Winkel. Daarna vertrok zij naar Dartmouth College, USA voor haar tweede stage op het microbiologie-laboratorium van prof.dr. R.J. Noelle. Na terugkomst in Nederland nam zij plaats in het bestuur van de A.U.S.R. Orca als commissaris vereniging. Na het behalen van het doctoraaldiploma eind 2001, begon zij begin 2002 als Onderzoeker In Opleiding bij de afdeling Moleculaire Celbiologie & Immunologie van het Vrije Universiteit medisch centrum onder begeleiding van dr. T.B.H. Geijtenbeek en prof.dr. Y. van Kooyk. De resultaten van het onderzoek naar de functie van de muis-homologen van DC-SIGN staan beschreven in dit proefschrift.

Appendix A

Color figures

Appendix	Reference
Figure A.1	Figure 4.3, pp. 73
Figure A.2	Figure 4.4, pp. 74
Figure A.3	Figure 6.1, pp. 100
Figure A.4	Figure 6.4, pp. 104
Figure A.5	Figure 7.1, pp. 116
Figure A.6	Figure 7.2, pp. 117

Table A.1: Reference table for reprinted color figures.

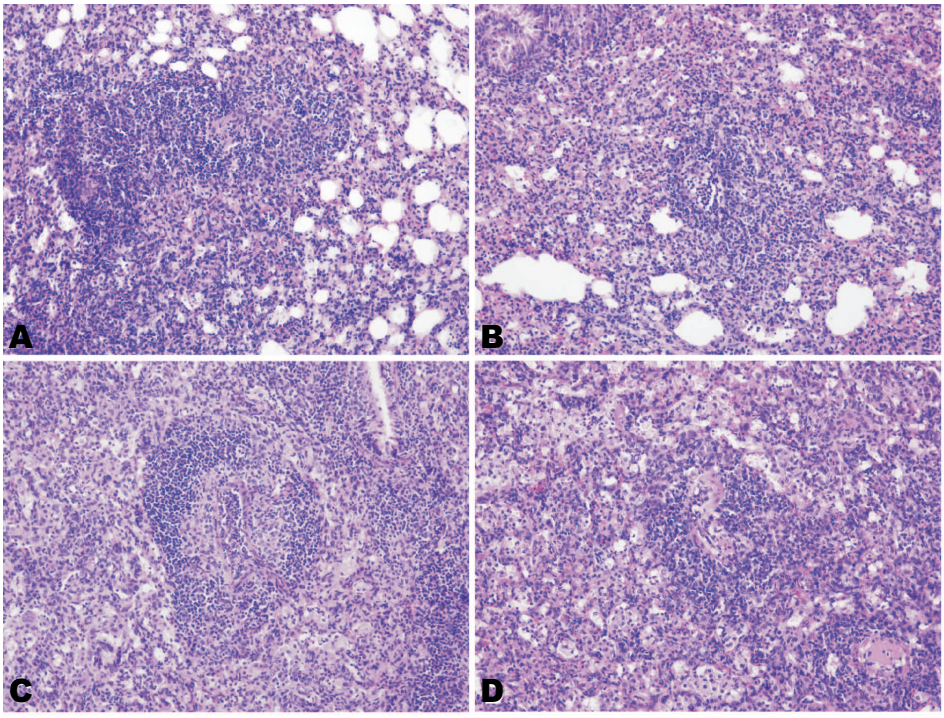

Figure A.1: In both WT and mSIGNR1 KO mice a similar degree of inflammation was observed in the lung
(color reprint of Fig. 4.3, pp. 73)
Representative lung histology of WT (A and C), and mSIGNR1 KO (B and D) mice, 2 (A, B) and 5 (C, D) weeks after intranasal infection with 10^5 CFU of *M. tuberculosis*. The lung sections are representative for 6-8 mice per group per time point. H&E staining, magnification $\times 10$.

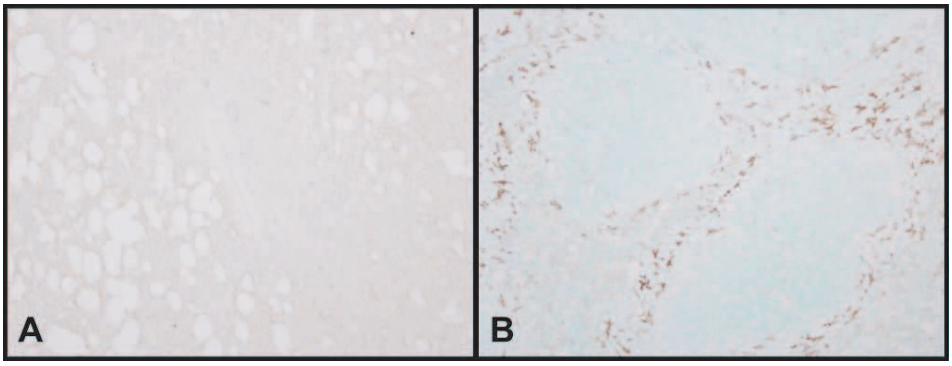

Figure A.2: No expression of mSIGNR1 in lungs of infected WT mice
(color reprint of Fig. 4.4, pp. 74)
Representative mSIGNR1 stainings of lung (A) and spleen (B) from WT mice 2 weeks after intranasal infection with 10^5 CFU of *M. tuberculosis*. No mSIGNR1 positive cells were detectable in the lung whereas marginal zone macrophages are stained positively. Sections are representative for 6-8 mice per group. Original magnification $\times 10$.

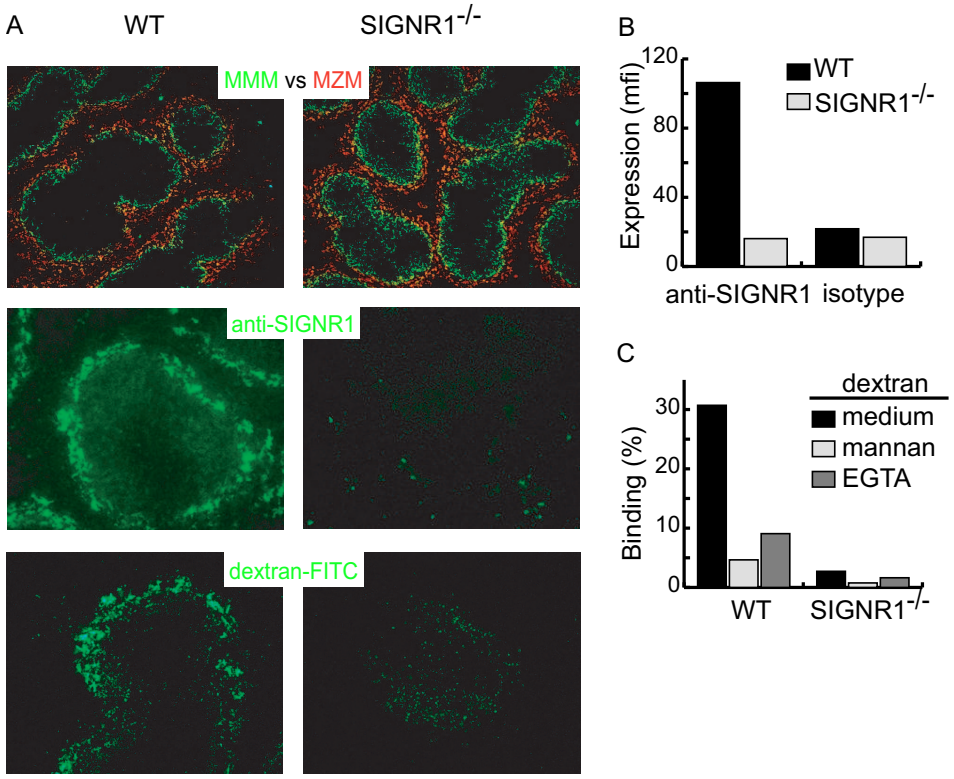

Figure A.3: mSIGNR1 captures blood-borne TI-II antigens in vivo
(color reprint of Fig. 6.1, pp. 100)
(A) The splenic marginal zone from SIGNR1$^{-/-}$ mice contains both marginal zone macrophage subsets (marginal zone macrophages (MZM) and marginal metallophilic macrophages (MMM) respectively), but does not capture TI-II antigens. Spleens from naive WT (left) and SIGNR1$^{-/-}$ (right) mice were stained for MARCO^{+} MZM in red and SER-4^{+} MMM in green (upper panel), for mSIGNR1 with ERTR-9 (middle panel) and for capture of dextran-FITC 45 minutes after intravenous administration (lower panel). These data are representative for three animals of both genotypes. (B and C) Peritoneal macrophages from SIGNR1$^{-/-}$ mice do not capture TI-II antigens. Peritoneal macrophages were analyzed for mSIGNR1 expression using ERTR-9 (B), and binding to dextran-FITC was analyzed in the presence and absence of the blocking reagents mannan and EGTA (C). These data are representative for three independent experiments.

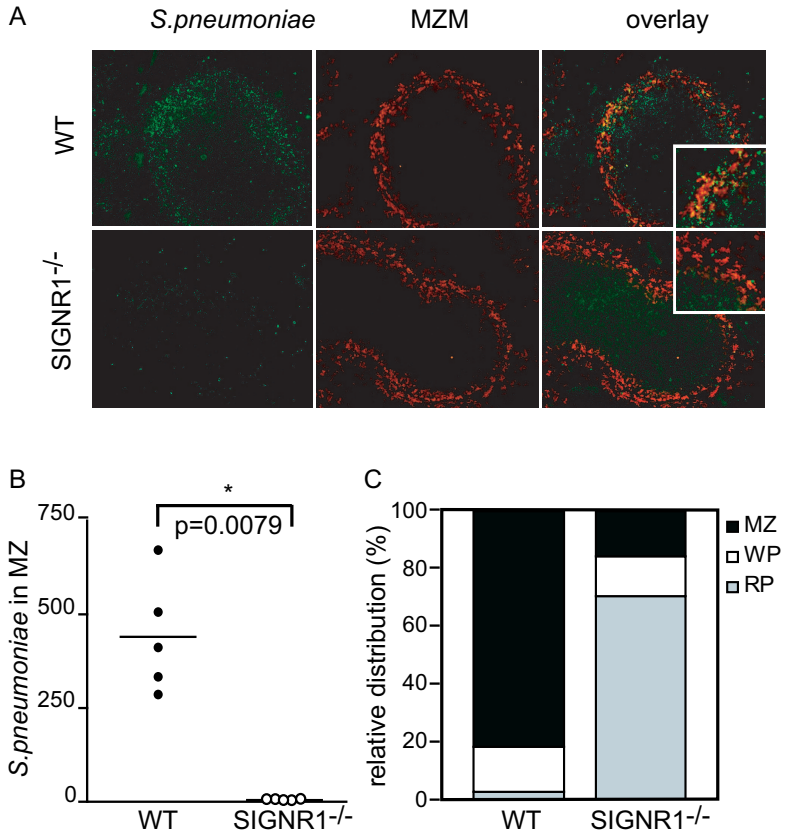

Figure A.4: mSIGNR1 is pivotal for the in vivo capture of blood-borne *S. pneumoniae* serotype 3 by the marginal zone

(color reprint of Fig. 6.4, pp. 104)

(A) *S. pneumoniae* is not captured in vivo by the splenic marginal zone in SIGNR1$^{-/-}$ mice. The capture of intravenous injected *S. pneumoniae* was determined in five representative visual fields of spleen sections of WT (upper) and SIGNR1$^{-/-}$ (lower panel) mice after 45 minutes. Spleens were stained for MARCO^{+} marginal zone macrophages (MZM) in red and *S. pneumoniae* in green. Inset shows pneumococci colocalizing and in close contact with WT MARCO^{+} MZM. Inset shows the absence of pneumococci in the marginal zone of SIGNR1$^{-/-}$ mice. (B) *S. pneumoniae* is captured by the marginal zone of the WT spleen whereas the pneumococci are hardly detected in the marginal zone of the SIGNR1$^{-/-}$ spleen. The amount of *S. pneumoniae* particles residing in the marginal zone of either WT or SIGNR1$^{-/-}$ mice was counted and compared. The capture of intravenously injected *S. pneumoniae* was determined in five representative visual fields of spleen sections of WT and SIGNR1$^{-/-}$ mice after 45 minutes. (C) The *S. pneumoniae* particles present in the SIGNR1$^{-/-}$ spleen reside mostly in the red pulp area (RP) of the spleen, in contrast to the *S. pneumoniae* particles present in the WT spleen which target to the marginal zone (MZ). These data are representative for three animals of both genotypes. $^{*}P < 0.05$.

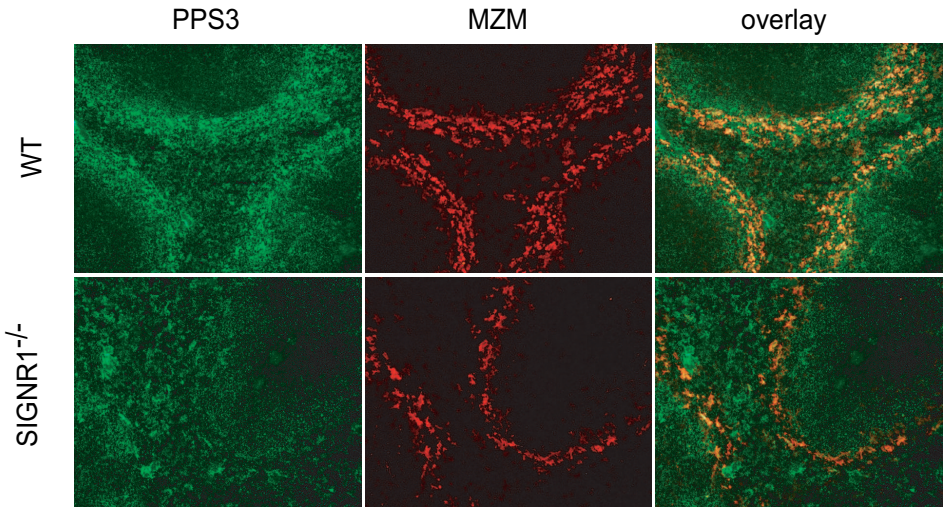

Figure A.5: mSIGNR1 is pivotal for the in vivo capture of blood-borne *S. pneumoniae* serotype 3 polysaccharide by the marginal zone
(color reprint of Fig. 7.1, pp. 116)
S. pneumoniae serotype 3 polysaccharides are retained by the marginal zone after intravenous injection in WT (upper) whereas it is dispersed throughout the spleen in mSIGNR1-deficient mice (SIGNR1$^{-/-}$, lower panel). Intravenously injected *S. pneumoniae* serotype 3 polysaccharide was detected in spleen sections of WT mice (upper) and mSIGNR1-deficient mice (lower panel) after 45 minutes. Spleens were analyzed for the expression of MARCO^{+} marginal zone macrophages (MZM) in red and pneumococcal polysaccharide serotype 3 (PPS3) in green.

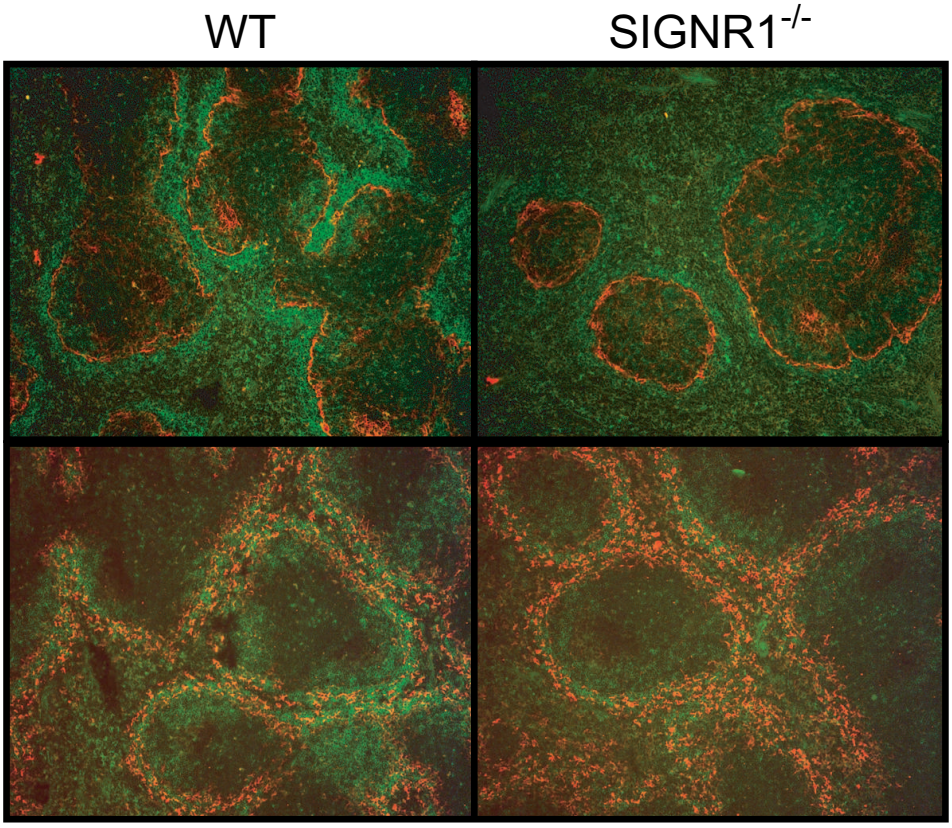

Figure A.6: Decreased numbers of marginal zone B cells in mSIGNR1-deficient mice
(color reprint of Fig. 7.2, pp. 117)
The splenic marginal zone from mSIGNR1-deficient mice contains a decreased amount of marginal zone B cells. Spleens from naive WT (left) and mSIGNR1-deficient mice (SIGNR1$^{-/-}$, right) were analyzed for the presence of MadCAM^{+} sinus-lining endothelial cells in red (upper panel) or MARCO^{+} marginal zone macrophages in red (lower panel) and the expression of CD1d in green. Marginal zone B cells were identified as the CD1d-postitive cells located outside the MadCAM^{+} rim and juxtaposed to the MARCO^{+} marginal zone macrophages. These data are representative for three animals of both genotypes.